UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

DIVISION OF HUMAN NUTRITION

DOCTOR OF PHILOSOPHY

Interaction of the hypothalamic-pituitary-adrenal axis with the serotoninergic anorectic D-Fenfluramine

Ross John Maclean

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ABSTRACT

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INTERACTION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS WITH THE SEROTONINERGIC ANORECTIC D-FENFLURAMINE

by Ross John Maclean

Arase et al. (1989) demonstrated that, in Fenfluramine-treated rats, adrenalectomy increased body weight loss and prevented the recovery of food intake that otherwise occurs 8-10 days after the beginning of drug treatment.

Results presented in this thesis show that the effects of adrenalectomy on fenfluramine action were eventually reversed if the animals were studied for longer than the 8-10 day period undertaken in the above study. This thesis describes the results of a study designed to investigate the effects of adrenalectomy on D-Fenfluramine (D-Fen) in the regulation of food intake and energy balance. The aim was to establish possible loci and mechanism(s) for these effects and to evaluate the results in terms of possible value for clinical usage.

The pharmacological and physiological systems associated with the modulation of D-Fen and adrenalectomy in the reduction of food intake and body weight were investigated.

The results presented in this thesis support the hypothesis that there is a potentiation of D-Fen action in adrenalectomised rats. The increased half-lives of D-Fen and D-Nor-Fenfluramine (D-Nor-Fen), following adrenalectomy, promotes increased body weight loss, a reduction in food intake, decreased efficiencies of food deposition, an increase in the activity of hypothalamic corticotropin releasing factor and of both the hypothalamic and hippocampal serotinergic system.

In conclusion, removal of corticosterone, by adrenalectomy, impairs the pathway for the metabolism and subsequent elimination of D-Fen and D-Nor-Fen. The pharmacological effect of D-Fen (and D-Nor-Fen) was enhanced, following adrenalectomy, since brain and serum levels of drug and metabolite remain elevated for a longer period.

The reversibility of the anorectic effects of fenfluramine observed by *Arase et al. (1989)* in intact animals is therefore retained in the adrenalectomised animals, however due to the mechanism postulated above, the time required for this to occur is increased.

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Finally, this thesis is for my parents. I finally finished it!

List of abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACTH	adrenocorticotrophic hormone
ADX	adrenalectomy
ANOVA	analysis of variance
ANS	autonomic nervous system
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CLIP	corticotropin-like intermediate lobe peptide
CNS	central nervous system
Cort.	corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione).
CRF	corticotropin-releasing factor
DA	dopamine
DAB	3,3'-diaminobenzidine (3,3',4,4'-tetraaminobiphenyl) tetrachloride
DEA	diethylaniline
5,6-DHT	5,6-dihydroxytryptamine
5,7-DHT	5,7-dihydroxytryptamine
DMN	dorsal motor nucleus of the vagus
D-Fen	D(+)-n-ethyl-a-methyl-*m*-trifluromethyl-phenethylamine (D-Fenfluramine)
D-Nor-Fen	D(+)-a-methyl-*m*-trifluromethyl-phenethylamine (D-Nor-fenfluramine)
ELISA	enzyme linked immunosorbant assay
Fen	D,L(±)-n-ethyl-a-methyl-*m*-trifluromethyl-phenethylamine (Fenfluramine)
GABA	gamma amino butyric acid
GAR	goat anti-rabbit
GC	gas chromatography
GEEFD	gross energetic efficiency of food deposition
GH	growth hormone
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTP	5-hydroxytryptophan (precursor of 5-HT)
HVA	3-methoxy-4-hydroxyphenyl ethyl alcohol / homovanillyl alcohol (HPLC internal standard)
ICC	immunocytochemistry
i.c.v.	intracerebroventricular (lateral ventricle)
i.m.	intramuscular
i.p.	intraperitoneal
IRMA	immunoradiometric assay
L-Fen	L(-)-n-ethyl-a-methyl-*m*-trifluromethyl-phenethylamine (L-Fenfluramine)
LH	lateral hypothalamus
L-Nor-Fen	L(-)-a-methyl-*m*-trifluromethyl-phenethylamine (L-Nor-Fenfluramine)
ME	median eminence

α-MSH	alpha melanocyte stimulating hormone
NAA	neutral amino acids
NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
Nor-Fen	D,L(±)-a-methyl-*m*-trifluromethyl-phenethylamine (Nor-Fenfluramine)
NPY	neuropeptide Y
PAP	peroxidase anti-peroxidase
PBS	phosphate buffered saline
PSNS	parasympathetic nervous system
PVN	paraventricular nucleus of hypothalamus
RIA	radioimmunoassay
s.c.	subcutaneous
SEM	standard error of mean
SNS	sympathetic nervous system
TBS	Tris-HCl buffered saline
VMH	ventromedial hypothalamus

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Chapter 1.

Introduction.

The physiological regulation of nutrient intake and energy expenditure is complex and multicompartmentalised. Distinct carbohydrate, fat and protein appetites regulate intake of energy. Alterations in these regulatory components can lead to changes in the energy balance of an individual.

The basic energy balance equation is summarised below:

ΔBody Energy = Energy Intake - Energy Expenditure

Failure to meet requirements for carbohydrate, fat and energy leads to a negative energy balance and weight loss. Oversupply of a particular nutrient or energy to requirement results in a positive energy balance and weight gain. Normally (in situations where growth, pregnancy, injury, or inflammation is not occurring) this equation is balanced so that energy intake equals energy expenditure.

The question that then arises is why do some individuals select an inappropriately high or low energy intake to their requirements?

This question can be answered by the study of the individual control mechanisms regulating protein, carbohydrate and fat intake together with the inter-relationships between the components. This will, hopefully, lead to a better understanding of the homeostatic processes occurring in the nutritional balance of an individual, and provide possible tools for the manipulation of nutrient intake in such situations as obesity or anorexia.

1.1. Peripheral regulation of nutrient balance.

1.1.1 Metabolic signals.

The regulation of body weight by circulating metabolic factors has been postulated (Bray *et al.* 1989). The role of these factors is discussed below.

Glucose.

The glucostatic theory postulates that the cause of satiation is mediated by the detection of the plasma glucose rise that occurs during the second half of a meal, and peaking at a meals end (Louis-Sylvestre and Le-Magnen 1980). Administration of glucose intra-gastrically causes a reduction in subsequent meal size, whilst 3-*O*-methylglucose has no effect (Booth 1972), which suggests that the effect of satiation is a metabolic and not an osmotic effect. Glucose alone is not sufficient to induce physiological satiety. Even and Nicolaidis (1986) infused glucose i.v. at 175% the rate of glucose utilisation, which resulted in a 40% reduction in food intake. The glucostatic theory may only really be demonstrating that glucose metabolism is monitored, and that glucose itself is not the causative factor in the control of satiation.

The lateral hypothalamus (LH) contains glucose-sensitive neurones (GSN's). The activity of the GSN's decreases in a dose-dependent fashion when their plasma membranes are exposed to glucose. The ventromedial nucleus of the hypothalamus (VMH) contains glucoreceptor neurones (GRN's). The GSN's and GRN's of the LH and VMH are involved in feeding control. Destruction of the LH and VMH with kainic acid destroys neurones in these regions, and spares fibres of passage, and results in a depression of feeding and obesity respectively (Grossman 1982, Shimizu *et al.* 1987), however small lesions of the VMH produce no alterations in food intake, and it is only if tracts lateral to the VMH are damaged that food intake is increased (Weingarten *et al.* 1988). The VMH is thought not to be a feeding centre, but an autonomic centre, and the resultant increase in food intake from VMH lesions is probably due to the damage of tracts from the PVN running lateral to the VMH. The GSN's and GRN's are also

sensitive to circulating levels of free fatty acids (oleic and palmitic acid), intermediate metabolites, insulin, glucagon, calcitonin, 5-HT and opioids (Oomura *et al.* 1992).

Insulin.

Feeding produces an initial surge of insulin, which is controlled to produce a maintained plasma concentration of glucose (Louis-Sylvestre and Le-Magnen 1980). Administration of insulin stimulates food intake and fat deposition with an accompanied hypoglycaemia. Insulin is an anabolic hormone that regulates the synthesis of proteins at the transcriptional, translational and the post-translational steps (Roy *et al.* 1980). Insulin modulates protein synthesis indirectly, also, by the promotion of branched chain amino acid (BCAA/NAA) uptake into muscle, inhibition of the oxidation of leucine and decreases protein breakdown (Hutson *et al.* 1978, Luck *et al.* 1928). Insulin, and similarly glucose, infusion cause significant reductions in serum levels of branched chain amino acids 20 minutes following injection (Schauder *et al.* 1983), due to a stimulation of peripheral amino acid uptake by muscle (Akedo and Christensen 1962). Obese patients, and animal models of obesity, display high insulin levels (Glass 1989, Bray 1989). This hyperinsulinaemia is a result of increased secretion of insulin as a direct result of increased islet tissue, increased vagal drive and an increase in sensitivity to glucose. Obese animal models and humans display decreased insulin receptor numbers in muscle (heart and skeletal), adipocytes and hepatocytes (Forgue and Freychet 1975, Olefsky *et al.* 1976, Olefsky 1976, Soll *et al.* 1975, 1976, Arner *et al.* 1983, Muggeo and Kahn 1981). Studies of insulin resistance in regional areas, utilising insulin clamp techniques, shows insulin resistance to be present in muscle (the most important area for insulin's amino acid uptake activity), adipocytes and hepatocytes (Defronzo 1982, Caballero 1987). Adrenalectomy reverses the muscle insulin resistance in *ob/ob* mice (Ohshima *et al.* 1984).

The insulin resistance has implications for the synthesis of brain 5-HT. The next section shows that the plasma levels of NAA effect regulation of food intake by altering the availability of precursors for 5-HT and catecholamines, and so affecting synthesis (Fernstrom and Faller 1978, Fernstrom and Wurtman 1972, Wurtman *et al.* 1980). Normally protein and carbohydrate ingestion stimulates amino acid uptake by

the muscle, with the sparing of tryptophan, and so the tryptophan:NAA ratio in plasma rises and increases CNS 5-HT by the increased availability of tryptophan (Fernstrom and Faller 1978, Fernstrom and Wurtman 1972, Wurtman *et al.* 1980). The reduction in plasma NAA (and hence the rise in the tryptophan/NAA ratio) following a carbohydrate or protein meal is blunted in the obese individual due to insulin resistance, leading to a lower tryptophan availability in the CNS and therefore less 5-HT activity. Caballero (1987), demonstrated obese humans consuming a 30g carbohydrate snack exhibited a blunted reduction in plasma NAA, despite the increased insulin response, compared to the lean humans displaying a reduction in the ratio tryptophan:NAA.

Amino acids.

Shifts in plasma amino acids caused by food ingestion provide signals to the brain that act in the control of feeding behaviour (Harper 1978). The signals that produce satiety from a high protein diet are acknowledged to be from the dietary amino acids (Harper *et al.* 1970). The protein selection of an animal is found to be reflected in the concentration of tryptophan in the plasma, expressed as a ratio to the other neutral amino acids (NAA), valine, isoleucine, leucine, tyrosine and phenylalanine (Ashley and Anderson 1975a/b, Fernstrom *et al.* 1971, 1978). A direct relationship is seen between the ratio tyrosine: phenylalanine and the tyrosine:NAA ratio (Anderson and Ashley 1977) to the amount of energy consumed.

The mechanism behind these observations regarding the control of feeding is thought to involve plasma amino acid uptake by the CNS. Uptake of each of these amino acids is determined by the individual plasma levels compared to the plasma concentration of the other NAA because of a general competitive transport for large NAA uptake into the brain (Blasberg and Lajtha 1965, Pardridge 1977). High plasma relative concentrations of tryptophan or tyrosine produce concomitant elevations in the CNS (Fernstrom and Faller 1978, Wurtman 1978, Wurtman 1987, Wurtman 1988). Fluctuations in brain tryptophan and tyrosine have effects on the synthesis of 5-HT and catecholamine levels respectively. The synthesising enzymes for 5-HT and catecholamines are not saturated at the range of amino acid concentration seen in the

brain (Fernstrom 1976, Gibson and Wurtman 1978) and the supply of precursors (tryptophan and tyrosine respectively) are the limiting factors in their formation. Carbohydrate intake increases the tryptophan:NAA ratio (Fernstrom and Wurtman 1971), and protein intake lowers the tryptophan:NAA ratio (Fernstrom *et al.* 1971), and consequently increases and decreases in brain 5-HT content respectively are seen (Anderson 1979). Other manipulations of tryptophan in the plasma by high tryptophan intake or by the removal of other NAA (Fernstrom and Wurtman 1972) increase CNS 5-HT, and conversely addition of branched chain amino acids (valine, isoleucine and leucine) decrease brain 5-HT (Fernstrom *et al.* 1976). Shifts in plasma tryptophan relative to the other large NAA effects the brain content of 5-HT and so provide regulation of protein consumption by alteration of food preference (Anderson 1979). The energy or total food intake is influenced by balances in the plasma tyrosine relative to the other large NAA, and the alterations in brain tyrosine and consequently the activity of catecholamines in the CNS is dependent on the uptake of tyrosine from the plasma (Anderson 1979, Caballero 1987, Holder and Heuther 1990).

Insulin, glucagon and corticosterone affect the relative and absolute concentrations of plasma amino acids. Insulin secretion, in a response to a meal, or by exogenous administration causes an increase in the ratio tryptophan:NAA, with a slight increase or no change in the total plasma tryptophan (Fernstrom 1976). Plasma tryptophan regulation of food intake is seen in the hyperphagic diabetic rat (*db/db*). Increasing the plasma tryptophan so that tryptophan:NAA approaches normal causes the excessive protein consumption to be reduced to normal (Caballero 1987). Plasma tyrosine:NAA is also low in the *db/db* mouse and the low tyrosine levels may be a factor in the increased energy hyperphagia by reducing noradrenaline synthesis in the CNS.

Section 1.1.3. deals with the importance of 5-HT and catecholamines in the regulation of food intake, and the plasma amino alterations seen with food intake and the change seen in the CNS clearly are important in the nutrient balance of the animals.

Fat metabolism.

Dietary fat is absorbed into the lymphatic system, rather than into the venous drainage of the GI tract, and so bypasses the liver. Intravenous infusion of emulsions of fat reduce food intake, an effect not accompanied by changes in plasma glucose or insulin (Woods *et al.* 1984). The inhibition of fatty acid utilisation with mercaptoacetate decreases the latency to eat, with no effect of meal size, which suggests fatty acid oxidation is involved in maintenance of satiety after a meal.

The fact that carbohydrate, fat and protein are all needed in i.v. infusion to completely suppress food intake (Nicolaidis and Rowland 1976), suggests that the peripheral signals for regulation of food intake are multiple rather than just a singular entity.

Gonadal factors.

Several congenital obesities such as Prader-Willi syndrome and Laurence-Moon-Biedl syndrome display testicular dysfunction. Serum testosterone levels are seen to be inversely proportional to the severity of obesity (Glass 1989). The low serum testosterone in obese males rises after weight loss (Strain *et al.* 1981, Strain *et al.* 1987). They display no hypogonadism, and the plasma free testosterone (thought to be the physiological active component of plasma testosterone) is increased, due to the reduction in plasma levels of sex hormone binding globulin (Strain *et al.* 1987).

Oestrogen production, in obesity, is increased, and the elevated oestrogen production would produce pituitary-hypothalamic suppression and hypogonadotropic hypogonadism. Adipose tissue can convert androgens to oestrogens (Perel and Killinger 1979), and so the increase in adipose tissue in obese men can explain the elevated serum oestrogen levels. Ovarian dysfunction is often associated with obesity (Hartz *et al.* 1987), and high serum levels of oestrogens and androgens are seen in obese post-menopausal women.

Conflicting results on leutinising (LH) and follicle-stimulating (FSH) hormones levels in obese humans exist (Kopelman *et al.* 1980, Kopelman *et al.* 1981, Kaufman *et al.* 1981), and the mechanism of the gonadal factors in obese women remain unclear.

Ovariectomy, in rats, produces obesity characterised by hyperphagia (Souquet and Rowland 1990), although the mechanism of action is uncertain.

Enterostatin

Enterostatin, a five amino acid sequence found in pancreatic procolipase, suppresses high-fat diet consumption following central and peripheral administration (Barton *et al.* 1995, Okada *et al.* 1993). The inhibition of high-fat intake by enterostatin is attenuated by opioid κ-receptor agonists (Barton *et al.* 1995).

Leptin

Leptin, a 167 amino acid hormone, exclusively produced by fat cells (Zhang *et al.* 1994), was discovered following investigation into the identity of the abnormal gene responsible for obesity in the obese *ob/ob* mouse. The gene responsible for producing leptin is absent in the *ob/ob* mouse. Injection of leptin into normal and *ob/ob* mice produces a reduction in body weight by a combination of anorexia and increased BAT thermogenesis (Halaas *et al.* 1995, Levin *et al.* 1996). Conversely, obese humans have been shown to produce larger amounts of leptin than lean subjects (Considine *et al.* 1996, McGregor *et al.* 1996). Leptin crosses the blood-brain barrier to interact with specific receptors in the hypothalamus to reduce food intake and increase BAT thermogenesis (Tartaglia *et al.* 1995, Mercer *et al.* 1996, Banks *et al.* 1996). The ratio of leptin blood:CSF is disproportionally lower in obese subjects compared to lean individuals (Caro *et al.* 1996, Schwartz *et al.* 1996). Leptin reduces the amount of NPY synthesised and released in the hypothalamus and reduces the effect of NPY following release (Stephens *et al.* 1995, Smith *et al.* 1996)

Other peripheral effects.

Growth hormone (GH) is a polypeptide secreted by the adenohypophysis whose principal function is the stimulation of growth through the stimulation of somatastatin by the liver. GH plays a major role on controlling growth, and obese patients have increased body weight, and decreased basal levels of GH and display blunted increases in GH to pharmacological stimulus i.e. insulin, arginine, glucagon, L-DOPA, glucose and propranolol (Glass 1989). GH defects in obese patients are thought to be as a

consequence of the obesity, and not a cause of the obesity (Kalkhoff *et al.* 1970, Ball *et al.* 1972).

Prolactin is a polypeptide hormone synthesised and released by the adenohypophysis under control from hypothalamic dopamine. Hypothalamic dysfunction, which is causal to some of the obesities in humans and animal models, could cause hyperprolactinaemia due to a loss of inhibitory factors. Appetite regulation occurs in the hypothalamus (Bray *et al.* 1989), and as hyperprolactinaemia is an early indicator of hypothalamic dysfunction, the prolactin status of obese patients is of interest. Although obese patients show normal basal and 24 hour prolactin levels, they show decreased response to stimulation by factors such as insulin, thyrotropin-releasing hormone, sulpride and chloropromazine (Glass 1989).

1.1.2. Role of thermogenesis in body weight and energy regulation.

Obligatory thermogenesis/Maintenance Energy Requirement is the energy required for the maintenance of essential bodily functions. This energy expenditure comprises functions including protein turnover, ion balance i.e. the resting metabolic rate (RMR) and the obligatory component of the thermic effect of feeding (TEF). TEF is part obligatory (for nutrient conversion) and part facultative (a variable component from individual to individual and probably mediated by the sympathetic nervous system). Obligatory thermogenesis can be raised during growth, pregnancy, lactation, by tumours, disease, inflammation and hyperthyroidism. The role of thermogenesis (whether dietary-induced, cold-induced or the thermic effect of food) is important to the equation on page 1. Any changes in the levels of energy expenditure of an animal must be compensated by changes to the energy intake, otherwise the body energy stores side of the equation will increase or decrease depending on whether energy expenditure is lower than or exceeds energy intake respectively.

Facultative thermogenesis and obligatory thermogenesis together give the total metabolic rate of the animal, which is controlled rapidly by the nervous system. Thermoregulatory thermogenesis (shivering in muscle and non-shivering in brown

adipose tissue) balances neural control of heat loss mechanisms, and in energy balance, whilst the control of facultative thermogenesis in muscle (exercise-induced) and in BAT (diet-induced) balances the control of food intake. The obese rodent models show reduction in energy expenditure. DIT is deficient in the *fa/fa* rats (Holt *et al.* 1983), in the rat made obese by surgical lesioning of the VMH (Hogan *et al* 1985, Van der Tuig *et al.* 1985) or chemically (Sahakian *et al.* 1983) or by parasagittal cuts (Coscina *et al.* 1985, Romsos *et al.* 1987), and in obesity-prone old rats (Marchington *et al.* 1986). The obesity is independent of the hyperphagia, as obesity is still manifested when pair fed to lean animals, and therefore a metabolic adaptation is occurring in these animals to produce obesity. Neuronal circuitry damage is not thought to be an important contributory factor in the absence of DIT, as adrenalectomy restores DIT in BAT in the *fa/fa* rats (Marchington *et al.* 1983, York *et al.* 1985), in the parasagittal knife cut rats (Romsos *et al.* 1987) and in the obesity-prone old rats (Marchington *et al.* 1986, Rothwell and Stock 1986). Cold-induced non-shivering thermogenesis (NST), in BAT, and other organs, is reduced in *ob/ob* (Himms-Hagen 1985), *db/db* and MSG (Tokuyama and Himms-Hagen 1986) induced obesities. These animals are unable to activate DIT. These factors result in altered energy balance and so obesity results from the increase in energy available for storage, which is increased in some cases by the presence of hyperphagia (Himms-Hagen 1989). Rats with PVN lesion-induced obesity have reduced BAT thermogenesis, as do rats with obesity induced by high fat or sucrose diets (Weingarten *et al.* 1985, Fukushima *et al.* 1987). Reduced energy expenditure by thermogenesis plays a role in the obesity in rodent models. The *fa/fa* rat obesity, due to a reduction in the facultative component of energy expenditure (DIT), may be due to the failure of peripheral signals from the diet to stimulate SNS. The obesity in the *ob/ob* and *db/db* mice, due to a deficient DIT and NST, may be due, in part, to a defect in the hypothalamic regulatory control of internal body temperature.

The development of safe and efficacious β_3 receptor agonists for the treatment of obesity in humans has yet to be achieved, despite the extensive research performed by many pharmaceutical companies.

1.1.3. Gastrointestinal signals.

Gastric distension has been shown to be a factor in the regulation of food intake. Mechanical distension terminates meals by a negative feedback system, and it is this feedback that enables gastric stapling to succeed (i.e. reducing the volume of food that can be consumed). The vagus is the principal afferent nerve in processing this information (Smith *et al.* 1989, Garlicki 1990). Another primary consideration for the success of gastric stapling is the conscious discomfort experienced by patients due to the stomach distension from a smaller volume of food. The gastric peptide cholecystokinin (CCK) is thought to mediate this action in producing satiety by delaying the gastric emptying and so promoting the duration of efferent vagal tone by maintaining a gastric distension (McHugh and Moran 1986, Baile *et al* 1986). These vagal nerves have been traced to the tractus solitarius through the parabranchial nucleus to the lateral (LH) and paraventricular nucleus (PVN) of the hypothalamus (Gibbs and Smith 1986) via a NA mechanism (Myers and McCleb 1980). CCK is produced in the gut wall in animals during and after feeding, and an increase in plasma concentration is seen following feeding (Greenley and Thompson 1984, Antin *et al.* 1975). Treatment with CCK to rats with open stomach fistulas prevents feeding, which would otherwise continue for hours (Gibbs and Smith 1986). This evidence lead to the thought that CCK is the major humoral satiety factor (Antin *et al.* 1975). However, gastric vagotomy prevents the hypophagic reaction to peripherally administered CCK, and the general consensus is that CCK is not a peripheral satiety hormone but may play a part in the peripheral events that lead to satiety and the termination of feeding.

1.2. The Hypothalamic-Pituitary-Adrenal axis (HPA).

The synthesis of glucocorticoids by the adrenal cortex is controlled by a negative feedback loop to inhibit the production at the hypothalamic, pituitary and adrenal cortical level. Corticotrophin releasing factor (CRF) released by the paraventricular nucleus cells of the hypothalamus (PVN), (Filaretov and Filaretova 1985, Vale *et al.* 1981), is secreted into the portal blood supply of the median eminence to stimulate the release of adrenocorticotrophic hormone (ACTH) from the adenohypophysis (Cunningham *et al.* 1988, Gangong 1977, Swanson *et al.* 1983). Pro-opiomelanocortin (POMC) is the 31 Kdalton precursor peptide for ACTH, α- and β-melanocyte-stimulating hormone (α- and β-MSH), β-lipotropin and β-endorphin. POMC is found in the arcuate nucleus, adenohypophysis, the intermediate lobe of the pituitary and the medulla (Zakarian and Smyth 1982, Knigge and Joseph 1984). POMC cleavage yields ACTH and β-lipotropin. Further cleavage of the ACTH peptide produces α- and β-MSH and CLIP; further cleavage of β-lipotropin yields β-endorphin. Glucocorticoids inhibit transcription of the POMC molecule in the corticotrophs of the adenohypophysis, and so inhibit the stimulation of itself by the reduction of the synthesis and therefore the secretion of ACTH (and also β-lipotropin, β-endorphin and β-MSH), (Herbert *et al.* 1980). ACTH release into the systemic blood supply stimulates synthesis and release of glucocorticoids from the adrenal cortical cells. Glucocorticoids inhibit ACTH secretion (Jones *et al.* 1976), and the synthesis of hypothalamic CRF, as shown by the increase in CRF mRNA following adrenalectomy, and the subsequent reduction in CRF mRNA in corticosterone replacement therapy to the adrenalectomised rats (Eberwine and Roberts 1984, Kovács *et al.* 1986, Sawchenko 1987, Kovács and Makara 1988, Vale *et al.* 1983, Beyer *et al.* 1988).

The adrenals lie in the retroperitoneal space near the upper lobe of each kidney and consist of two separate endocrine organs. The inner region, the medulla, secretes the catecholaminergic hormones, adrenaline and noradrenaline. The outer region, the cortex, secretes steroid hormones, primarily the glucocorticoids, and mineralocorticoids which regulate sodium balance and extracellular fluid volume. The adrenal cortex has only sparse nervous innervation, which are mostly associated with the blood vessels,

whilst the medulla contains dense nervous innervation by preganglionic cholinergic fibres in the splanchnic nerve. Circulating ACTH stimulating adenylate cyclase in the cells of the zona fasiculata and zona reticularis controls secretion of glucocorticoids. The increased production of cAMP stimulates steroid synthesis by activating glycogenolysis, increasing the availability of glucose-6-phosphate (G-6-PO_4) and stimulating enzymes involved in steroid synthesis (G-6-PO_4 dehydrogenase), which diverts G-6-PO_4 into the pentose phosphate pathway and so increases the amount of NADPH. NADPH is involved in the hydroxylation step of steroid synthesis, which includes the rate-limiting step of steroid biosynthesis, the conversion of cholesterol of pregnenalone in the mitochondria.

The adrenal gland has no storage mechanism for glucocorticoids, therefore the stimulation of synthesis is accompanied by an increase in release. Injection of ACTH stimulates the synthesis and output of glucocorticoids within 2 minutes, and removal of the pituitary (to remove endogenous ACTH) causes a decline of glucocorticoids secretion to low levels within 60 minutes. In the absence of a stimulatory process to the hypothalamic-pituitary-adrenal axis (i.e. stress, trauma), a diurnal rhythm of ACTH and glucocorticoid secretion is seen. In man, peak glucocorticoid secretion is usually at 0700 hours, falls rapidly over 2-3 hours and then slowly declines to its lowest level in early evening. This rhythm slowly adapts to changes to the normal sleep/awake cycle i.e. international travellers, night-shift workers, and is controlled by activity in the limbic lobes of the cerebral cortex. In rats a circadian rhythm of glucocorticoid secretion, in this case corticosterone, is observed, but with peak corticosterone seen in the early evening, with low levels at the start of the light cycle.

1.2.1. Role of glucocorticoids.

Glucocorticoids promote the mobilisation of proteins and amino acids firstly from skeletal muscle and then from other tissues such as the bone matrix and skin. Amino acids and proteins are transported to the liver. Glucocorticoids also stimulate the enzymes of gluconeogenesis and glycogenesis (glucose-6-phosphatase and PEP carboxylase), (Exton *et al.* 1979). Amino acids are then converted to glucose and liver

glycogen storage. There is a net negative nitrogen balance. Glucocorticoids reduce insulin secretion and so reduce glucose and amino acid uptake, promoting the previous effects.

Glucocorticoids exert a permissive role in relation to lipolysis induced by other hormones, but have little direct lipolytic activity themselves. The mechanisms of action in terms of fat mobilisation and metabolism are not understood. Glucocorticoids need to be present for the storage of fat in the liver and production of ketone bodies from fat intake and/or from a decrease in insulin secretion. The role of glucocorticoids in fat mobilisation and distribution is shown by the redistribution of fat in the neck and shoulder region (buffalo hump) and in the cheeks (moon face), coupled with the loss of fat from the extremities, seen in people with exposure to high levels of glucocorticoids i.e. Cushing's syndrome, glucocorticoid treatment, which also produces hyperglycaemia and a reduction in peripheral utilisation of glucose, often producing insulin-resistant diabetes mellitus, conversely, people with a deficiency in glucocorticoids (Addison's disease) are usually very thin with losses in peripheral fat stores.

Other effects of glucocorticoids include osteoporosis, due to the excessive protein catabolism inhibiting new bone formation, the antagonism of vitamin D and the increase in glomerular filtration rate observed with high levels of glucocorticoids increasing the level of urinary excretion of calcium. Adrenalectomy produces increased capillary permeability, reduced blood pressure (due to impaired reactivity of blood vessels to noradrenaline) and reduced cardiac output. Heart muscle atrophy and a reduction of heart contractility occurs. Excessive glucocorticoids result in hypertension. Glucocorticoids reduce the number of circulating eosinophils, basophils and lymphocytes by a breakdown in the thymus, lymph nodes and spleen, and an inhibition of division of the remaining lymphocytes, and an increase in the number of circulating neutrophils and erythrocytes. Reticuloendothelial amoeboid and phagocytic cell activity is suppressed by glucocorticoids. These factors produce a net suppression of the inflammatory response of an animal.

Glucocorticoids produce many central effects (Funder and Sheppard 1987). Corticosterone crosses the blood brain barrier (Pardridge and Mietus 1979), and glucocorticoid receptors are located in many areas of the CNS, mostly densely in the hippocampus, and are also located in the hypothalamus and cerebellum (Meyer 1985).

Adrenalectomy increases noradrenaline turnover in the hypothalamus and replacement therapy with hydrocortisone abolishes the increase (Fuxe *et al.* 1973). Tyrosine- and dopamine- β-hydroxylase enzymes are stimulated by corticosterone (Markey *et al.* 1982, Meyer 1985), and circadian variations of the activities of these enzymes exist (in conjunction with the circadian rhythm of corticosterone), (Cahill and Ehret 1981).

5-HT synthesis is reduced with corticosterone treatment (Curzon and Green 1982, Green and Curzon 1968, Shah *et al.* 1968). A possible method for the reduction in brain 5-HT activity is the induction of hepatic tryptophan pyrollase by glucocorticoids, and therefore a limitation in the availability of tryptophan into the CNS (Meyer 1985). However, Yuwiler *et al.* (1971, 1978) found no relationship between the activity of the enzyme tryptophan pyrollase and brain 5-HT levels. Long-term adrenalectomy increases brain tryptophan, with plasma tryptophan being unaltered and brain indoleamines are in fact being reduced (Miller *et al.* 1978). Betamethasone reduces 5-HT and 5-HIAA in rat hippocampus (De Kloet *et al.* 1983, Balfour *et al.* 1979) and not the hypothalamus (Balfour *et al.* 1979). Others have demonstrated adrenalectomy to have either no effect on brain 5-HT (Shah *et al.* 1968) or a reduction (Kovács *et al.* 1977, Rastogi and Singhal 1978, Telegdy and Vermes 1975). The changes in 5-HT (where occurring) are not apparent until one week following adrenalectomy (Rastogi and Singhal 1978). 5-HT turnover, measured by Van Loon (1981,1982), showed adrenalectomy-reduced turnover in the hypothalamus and hippocampus, which was reversible by corticosterone treatment. Long *et al.* (1983) have shown a non-corticosterone reversible reduction in 5-HT in hypothalamic median eminence and nucleus centralis superior. 5-HT uptake by hypothalamic synaptosomes is reduced by 30% in adrenalectomised animals (Vermes *et al.* 1976). Corticosterone treatment prevents this reduction. Increased release of 5-HT from hypothalamic synaptosomes (by depolarisation) occurs after adrenalectomy. It seems that glucocorticoids increase

uptake and inhibit release from nerve terminals. The influence of corticosteroids on hippocampal 5-HT is via a corticosterone receptor mechanism. Corticosterone receptor binding is low in the raphe area (De Kloet *et al.* 1983), the source of hippocampal afferents, so it is postulated that there might be a form of negative feedback to the raphe from the hippocampus (Kimble 1968), or a self-regulating system of hippocampal post-synaptic systems involving 5-HT neurones via a corticosterone receptor mechanism.

Glucocorticoids stimulate choline uptake by a high affinity Na^+-dependent uptake mechanism (Sze *et al.* 1983, Riker *et al.* 1979) although choline uptake by rat hypothalamic synaptosomes is not affected by adrenalectomy (Vermes *et al.* 1976). The role of glucocorticoids in acetylcholine (ACh) regulation in the brain has not been elucidated.

Glucocorticoids do not affect either glutamate decarboxylase, the enzyme responsible for gamma amino butyric acid (GABA) synthesis or GABA aminotransferase, the enzyme that catalyses the first step in GABA metabolism (Meyer *et al.* 1979). Although, hippocampal synaptosomes from adrenalectomised rats show increased GABA uptake, which is reversed by corticosterone replacement therapy (Miller *et al.* 1978). Adrenalectomy causes corticosterone reversible increases in GABA binding to corpus striatum and midbrain, but not to hypothalamus, hippocampus, cerebellum, cerebral cortex or brain stem (Kendell *et al.* 1968). Adrenalectomy also fails to change the concentration of GABA or high affinity GABA uptake in any of the brain areas. The causes of the differential effects of GABA are unclear.

Enkephalin brain levels show no changes following corticosterone treatment (Gibson *et al.* 1980). Adrenalectomy reduces vasoactive intestinal peptide (VIP) and neurotensin in the hippocampus and adenohypophysis. Corticosterone replacement therapy reverses the reduction only in the hippocampus (Meyer 1979), whilst dexamethasone restores VIP in all the areas (Meyer 1979).

1.2.2. Corticotropin Releasing Factor (CRF).

In 1981 Vale *et al.* isolated the hypothalamic ACTH-releasing factor. It was found to be a 41 amino acid peptide (Mw 4757.5) showing structural homologies with a vertebrate peptide, sauvagine (isolated from the skin of *Phyllomedusa sauvagei*) and urotensin-I (isolated from the urophysis of two separate species of sucker fish), (Morley and Kay 1986). CRF for many species have now been elucidated. The inter-species homology of CRF is presented below.

Structural homologies between mammalian CRF (rat/human/porcine/avian/bovine/ovine), urotensin-I and sauvagine.

CRF (rat/human)	H-SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII-NH_2
CRF (porcine)	H-SEEPPISLDLTFHLLREVLEMARAEQLAQQAHS**H**RKLME**NF**-NH_2
CRF (avian)	H-S**Q**EPPISLDLTFHLLREVLEM**TK**A**D**QLAQQAHSNRKL**LD**I**A**-NH_2
CRF (bovine)	H-S**Q**EPPISLDLTFHLLREVLEM**TK**A**D**QLAQQAH**N**NRKL**LD**I**A**-NH_2
CRF (ovine)	H-S**Q**EPPISLDLTFHLLREVLEM**TK**A**D**QLAQQAHSNRKL**LD**I**A**-NH_2
Sauvagine (*Phyllomedusa*)	**pEG**PPIS**I**DL**SLE**LLR**KMI**E**IEKQQKEK**QQA**AN**NRL**LL**DT**I-NH_2
urotensin-I (sucker fish)	H-**NDD**PPIS**I**DLTFHLLR**NMI**EMAR**I**E**NERE**QA**GL**NRK**YLDEV**-NH_2

From Vale and Greer 1984, Fisher 1989. One letter amino acid abbreviations (Biochemistry, Stryer, Second Ed.). Bold/underlined amino acids denotes differences from rat/human CRF_{41}.

CRF causes a dose-related release of pituitary adrenocorticotropic hormone (ACTH) and β-endorphin *in vivo* and *in vitro* (Vale *et al.* 1981). A hypothalamic-infundibular CRF pathway is involved in this CRF mediation of ACTH and β-endorphin release, via the hypothalamic-hypophyseal portal blood system (Swanson *et al.* 1983, Merchenthaler *et al.* 1984). ACTH release from the adenohypophysis stimulates the release of glucocorticoids from the adrenal cortex (see section 1.1.2.). The parvocellular neurosecretory neurones responsible for the synthesis and delivery of CRF to the median eminence for stimulatory control of ACTH release from the adenohypophysis are located in the PVN region of the hypothalamus (Antoni *et al.* 1983, Agnati *et al.* 1985, Merchenthaler *et al.* 1983b, Swanson *et al.* 1983). CRF containing neurones play an important role in modulating the stress response by projections to the medien emminence, brain stem and spinal. CRF also possesses extrahypothalamic actions to the potent stimulatory action of ACTH, including inhibition of food intake and stimulation of the sympathetic nervous system and energy expenditure.

Intracerebroventricular injections of CRF lead to autonomic and behavioural responses (Brown *et al.* 1982, Morley and Levine 1982, Holt *et al.* 1989, Egawa *et al.* 1990). CRF reduces feeding (Arase *et al.* 1988) by shifting the animals behaviour to an increase in grooming (Morley and Levine 1982). CRF reduces feeding induced by muscimol, NA, dynorphin and insulin (Levine *et al.* 1983), and potently inhibits feeding in hypophysectomised rats, which indicates that the effects are not secondary to CRF effects on pituitary ACTH secretion (Morley and Levine 1983). CRF inhibition of food intake can be antagonised with treatment with an oxytocin antagonist [$\alpha(CH_2)_5$, $Tyr(Me)^2$, Orn^8] vasotocin, suggesting a possible oxytocin mediated CRF-induced anorexia (Olson *et al.* 1991). CRF decreases carbohydrate intake while not affecting other components of dietary intake, not unlike the action of central 5-HT and D-Fen (Morley *et al.* 1986), although low doses of CRF have been shown to reduce fat intake whilst carbohydrate intake is only reduced at high doses of CRF (York, personal communication).

CRF fulfils the criteria necessary to be the primary hypothalamic factor governing the pituitary-adrenal axis (Rivier and Plotsky 1986), by:

- Potently stimulating ACTH, and POMC-cleaved peptides (*in vitro* and *in vivo).*
- CRF immunoreactive staining is present in the hypophysiotropic zone of the hypothalamus, as well as in the hypophyseal portal blood (Hiroshige *et al.* 1977).
- High-affinity CRF binding sites (Kd = 1nm) in the pituitary, (Hauger *et al.* 1988).
- Immunisation of animals with anti-CRF sera/synthetic CRF antagonists attenuates or abolishes stress induced ACTH secretion.
- CRF neurones mediating hypophyseal functions located in the PVN. These cells project into the external layer of the ME, and terminate on hypophyseal portal blood vessels.

Approximately 2000 cells stain positive to CRF in the paraventricular nucleus, with the densest region in the medial parvocelluar region. CRF is also located throughout the brain and spinal cord, shown by radioimmunological and immunohistochemical staining (Swanson *et al.* 1983). Positive staining has been shown in the corpus

callosum, hippocampus, septal region, anterior commissure, bed nucleus of the stria terminalis, substantia innominata, central nucleus of the amygdala, medial preoptic area, median eminence (ME), posterior pituitary, lateral hypothalamus (PFN), medial forebrain bundle, locus coeruleus, midline thalamic nuclei, stria terminalis, perioculomotor nucleus, central grey, dorsal raphe, median raphe, laterodorsal tegmental nucleus, locus ceruleus, parabranchial nucleus, medial vestibular nucleus, dorsal motor nucleus of the vagus, A_5/A_1 NA cell groups, prepositus hypoglossal nucleus, external cuneate nucleus, inferior olive and the medullary reticular formation. In particular, the median eminence, lateral septum, bed nucleus of the stria terminalis, mesencephalic reticular formation, parabranchial nuclei and nucleus of the solitary tract contain large numbers of CRF immunoreactivity also (Sakanaka *et al.* 1987, Ceccatelli *et al.* 1989, Cummings *et al.* 1983, Swanson *et al.* 1983, Skofitsch and Jacobwitz 1985, Aguilera *et al.* 1987, Berkenbosch *et al.* 1986, Merchanthaler *et al.* 1982, 1983a/b, Olschowa *et al.* 1982a/b, Palkovits *et al.* 1985).

The localisation of CRF containing neurones in regions other than the hypophyseal zone, has developed interest in CRF's potential role as a neurotransmitter or neuromodulator in the CNS. Several results suggest a neurotransmitter status of CRF:

- Depolarising agents (K^+ and snake venom) cause a release of CRF from brain regions, which is Ca^{2+} dependent (Smith *et al.* 1986).
- CRF receptors show widespread and non-uniform distribution, with high affinity (Kd=0.2nm). These receptors are adenylate linked (as in CRF receptors in the pituitary), but the second messenger systems and/or the regulation of these receptors may be different (Fisher 1989). Evidence for this is shown by the apparent different modulations between brain and pituitary receptors. Following adrenalectomy there is a selective increase in adenohypophyseal, medium eminence and PVN (the anterior and medial paricellular subdivisions particularly) CRF immunoreactivity and CRF synthesis, but returns to pre-adrenalectomy levels within 5-7 days (Sawchencko *et al.* 1983, Kovács *et al.* 1986, Jingami *et al.* 1985, Kiss *et al.* 1984, but other brain regions show no change in the number of CRF receptors (Koob and Bloom 1985).

Glucocorticoids act directly on the PVN to inhibit CRF secretion. Glucocorticoid administration to the PVN region results in an abolition of the increase in CRF immunoreactivity and increased CRF mRNA observed following adrenalectomy (Jingami *et al.* 1985, Kovács *et al.* 1986). This agrees with the observation of high nuclear concentration of immunohistochemical reactivity to rat liver glucocorticoid receptor within the PVN, with a distribution highly correlated to CRF immunoreactivity (Agnati *et al.* 1985). The involvement of CRF on the autonomic nervous system output and function of peripheral organs are important observations, and because of the involvement of stressful stimuli in stimulating the activity of the pituitary-adrenal axis and sympathetic nervous system activity, any alteration to the CRF balance may produce alterations in the response to stress.

CRF and the Autonomic Nervous System (ANS).

CRF, administered intracerebroventrically (i.c.v.), intracisternally (i.c.) or intraparenchymally stimulates the sympathetic nervous system (SNS), and adrenomedullary activity (Brown *et al.* 1982, Brown and Fisher 1983, Brown and Fisher 1985, Holt *et al.* 1983). These effects are mediated centrally, as the plasma elevations of catecholamines are comparable to animals pre-treated with i.v. administration of anti-CRF (Brown and Fisher 1985). The site of CRF action to stimulate SNS, by increasing NA levels, is thought to be multiple, as this effect is seen from multiple injection sites (Brown 1986). The proposal of multiple sites is supported by the wide distribution of CRF immunoreactivity within the CNS, and the similar observations of the distribution of CRF receptors within the limbic, hypothalamic, brainstem and spinal cord areas, which are associated with the regulation of the autonomic nervous system. CRF, centrally administered inhibits vagal tone (Brown *et al.* 1982) and acts centrally to increase the activity of efferent adrenal nerves (Geiger and Nagy 1985) and sympathetic firing to BAT (Holt and York 1989). The use of α-helical CRF (CRF_{9-41} or CRF_{10-41}) i.c.v. reduces the rise in plasma adrenaline associated with stress (Brown *et al.* 1985, Brown and Fisher 1985).

CRF and Central Neurotransmitters.

The neurotransmitter regulation of CRF secretion has been studied widely, and evidence shows that central acetylcholine, noradrenaline, the opioids and 5-HT are all involved in the secretion of CRF. Procaine and lignocaine both stimulate CRF secretion (Calogero *et al.* 1990). Section 1.1.3. deals with the importance of central neurotransmitters involved with the modulation of nutrient intake.

Noradrenaline inhibits central CRF release (Jones and Hillhouse 1977, Suda *et al.* 1987, Szafarczyk *et al.* 1988). This effect is via an α_1 adrenergic receptor, and is abolished by phentolamine (Yajima *et al.* 1986). Further evidence to support this inhibitory effect is by the presence of catecholaminergic input neurones to CRF containing cells in the PVN (Kitazawa *et al.* 1987). GABA also inhibits CRF release (Jones and Hillhouse 1977).

Melatonin, one of the metabolites of 5-HT, inhibits CRF secretion (Jones and Hillhouse 1977), and also inhibits the 5-HT and ACh stimulation of CRF. The inhibition of both ACh and 5-HT induced CRF secretion suggests that the inhibitory action of melatonin is not due to an antagonist action on 5-HT receptors, but via a direct action on CRF neurones. The circadian variation in 5-HT release may underline the circadian control of CRF and glucocorticoid levels.

In humans, opioid peptides inhibit the release of ACTH and cortisol secretion (Stubbs *et al.* 1979). In rats, opioids inhibit ACTH and corticosterone secretion. In vitro perfusion of rat hypothalamus with β-endorphin and dynorphin suppresses CRF secretion (Yajima *et al.* 1986). These effects are inhibited by naloxone, suggesting that opioid peptides act through opioid receptors to inhibit CRF.

5-HT is implicated in both the maintenance of the circadian rhythm of plasma ACTH and the modulation of ACTH to stress (Vernikos-Danellis *et al.* 1974, 1977).

Serotoninergic drugs that increase the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Calogero *et al.* 1989, McElroy *et al.* 1984) include precursors, 5-HTP

[L>D] (Popova *et al.* 1972, Fuller *et al.* 1975a, 1976), 5-HT releasers, p-chloroamphetamine (Fuller and Snoddy 1980), Fen (Fuller and Snoddy 1980, McElroy *et al.* 1984), Nor-Fen (Fuller and Snoddy 1980) and 3,4-methylenedioxy-methamphetamine (Nash *et al.* 1988), 5-HT uptake inhibitors, fluoxetine (Fuller *et al.* 1976), ORG 6582 and p-bromo EXP 561 (Fuller 1980b), direct 5-HT agonists, quipazine (Fuller *et al.* 1978a), *m*-trifluromethyl-phenylpiperazine (Fuller and Snoddy 1979), *m*-chloropheny-piperazine (Fuller *et al.* 1981), MK-212 (Koenig *et al.* 1988), RU 24969 (Euvrard and Boissier 1980), 8-OH DPAT (Gilbert *et al.* 1988a/b, Nash *et al.* 1988), buspirone, gepirone and ipsapirone (Koenig *et al.* 1988). Systemic and peripheral administration of 5-HT stimulate the HPA axis (Abe and Hiroshige 1978, Di Renzo *et al.* 1978, Fuller *et al.* 1976).

5-HT causes a dose related release of CRF from hypothalamic cultures in vitro (Calogero *et al.* 1989, Jones *et al.* 1976) and in vivo (Gibbs and Vale 1983), and this effect is mimicked by addition of chlorimipramine or D-Fen. The D-Fen stimulation of CRF is greatly reduced in the presence of the 5-HT receptor antagonists, methylsergide and metergoline (Nakagami *et al.* 1986, Jones and Hillhouse 1977, Holmes *et al.* 1982). This effect is also blocked by hexamethonium (Jones and Hillhouse 1977). The stimulation of CRF by 5-HT in explanted hypothalamus is thought to be mediated by 5-HT_2 receptors (Calogera *et al* 1989). The role of 5-HT (and related compounds) in food intake and thermogenesis has been postulated to involve the release of CRF, and immunisation against CRF inhibits the thermogenic and anorexic actions of 5-HT agents (5-HT, 5-HTP and D,L-Fen), (Le Feuvre *et al.* 1987).

5-HT-containing nerve terminals make axodendritic and axosomatic synapses with CRF containing neurones within the PVN (Liposits *et al.* 1987). The origin of 5-HT terminals is uncertain, but 5-HT projections to the PVN have been reported to originate from the B_7, B_8 and B_9 nuclei of the midbrain (Sawchenko *et al.* 1983, Van de Kar *et al.* 1982, 1985). Lesions of dorsal or median raphe nucleus do not affect stimulation of plasma corticosterone by p-chloroamphetamine, whilst the possibility of a 5-HT intrahypothalamic network may exist for the 5-HT stimulation of the HPA (Fuller 1990, Tork 1985).

A possible role for 5-HT in the regulation of the HPA axis is the negative feedback of glucocorticoids by ACTH. Adrenalectomy (in some reports) changes the metabolism of 5-HT (Van Loon *et al.* 1982) and corticosterone mediated behaviour (Telegdy *et al.* 1975). Adrenalectomy increases the density of 5-HT_1 receptors in the dorsal raphe nucleus, hippocampus and hypothalamus, which returns to normal after corticosterone replacement therapy (De Kloet *et al.* 1983). Glucocorticoid receptors are found in the raphe. These results suggest that 5-HT neurones from the dorsal raphe and/or nerve terminal in the hippocampus and hypothalamus regulate feedback of ACTH secretion. The 5-HT may be activating post-synaptic 5-HT_{1A} receptors on CRF synthesising neurones in the PVN (Liposits *et al.* 1987), as PVN lesions, using 5,7-DHT, prevent the increase in plasma corticosterone by 5-HT stimuli (Feldman *et al.* 1987), and together with the other anatomical and pharmacological data, suggests 5-HT terminals in the PVN stimulate CRF secretion by direct synaptic activation. Hippocampal 5-HT receptors are regulated by corticosterone (Chaouloff 1995). Control is exerted at the level of the 5-HT_{1A} receptor gene. Information on the role of corticosterone and the control of other receptors (5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2C}) is lacking or contradictory.

ACh, choline acetyltransferase and acetylcholinesterase are present in the hypothalamus (Jones and Hillhouse 1977). Many hypothalamic nuclei contain choline acetyltransferase, with the largest concentration being found in the arcuate, medium eminence, dorsomedial and paraventricular nuclei (Uchimura *et al.* 1975, Jones and Hillhouse 1977). ACh dose-dependently stimulates CRF release from hypothalamic nuclei and other brain area cell cultures (Jones and Hillhouse 1977, Hillhouse and Reichlin 1990), which is mediated by a mixed receptor system, which is primarily nicotinic as hexamethonium fully blocks the response, whilst atropine only partially blocks. The pure muscarinic agonist bethanocol does not stimulate CRF secretion (Jones and Hillhouse 1977, Tsagarakis *et al.* 1988).

CRF and Obesity.

Low levels of pituitary mRNA for pro-opioimelanocortin (POMC), the precursor for several CNS peptides, including ACTH, and reduced levels of PVN CRF are seen in obese rats (Nakai *et al.* 1989, Beyer *et al.* 1988). Apparent low levels probably reflect increased secretion in these animals (i.e high corticosterone levels). Obese rats are more responsive to the actions of CRF than lean counterparts (Arase *et al.* 1988). The alterations in the CRF status of obese rodent models may be a contributory factor in the manifestation of obesity, other than the hyperphagia component. The increases in PVN CRF associated with adrenalectomy, the reversal of obesity following adrenalectomy and the reduced sympathetic activity of obese rats compared to lean animals (which is increased by CRF treatment centrally) point to an important interaction between CRF, the hypothalamic-pituitary-adrenal axis and the autonomic nervous system in the regulation of the energy balance in obesity (see section 1.1.5.). Further interactions are seen with other neurotransmitters are also apparent, (Vernikos-Danellis *et al.* 1974, Vernikos-Danellis *et al.* 1977).

1.3. Central regulation of nutrient balance.

Many regions of the brain display a role in the control of food intake and energy balance, with the hypothalamus being particularly important. Destruction of brain areas using electrolytic, neurotoxic or mechanical lesioning, or stimulation electrically or by application of known agonists, enables the function of specific brain regions in energy balance regulation to be studied.

Noradrenaline, 5-HT, histamine, GABA and many peptides are seen to be involved in the regulation of food intake and energy balance (Chance 1987, Leibowitz 1987, Samanin and Garattinni 1982, Samanin *et al.* 1978).

1.3.1 Stimulatory neurotransmitters.

Noradrenaline/adrenaline

Noradrenaline (NA), with adrenaline (ADR), stimulate food intake via α_2-noradrenergic receptors, located specifically in the PVN region of the hypothalamus (Leibowitz and Brown 1980a/b, Kow and Pfaff 1989). The NA innervation to the PVN region, which is involved in the control of appetite, originates primarily from a variety of NA containing cells of the lower brain stem (dorsal component of central tegmental tract), (Hökfelt *et al.* 1974, Maeda and Shimizu 1972, Moore and Bloom 1979, Olsen and Fuxe 1972).

Physiological doses of noradrenaline injected into the PVN of satiated rats causes a feeding response within 2 minutes (Booth 1968, Leibowitz 1975a, Leibowitz 1978a). Chronic infusion of NA or clonidine causes an increase in daily food intake and body weight (Leibowitz *et al.* 1984). 6-OH DA injection into the PVN, to destroy PVN noradrenergic innervation decreases daily food intake and body weight gain (Leibowitz and Shor-Posner 1986b). As previously mentioned, PVN lesioning produces hyperphagia and obesity (Leibowitz *et al.* 1981), and abolishes the action of NA injection into this region (Leibowitz *et al.* 1983). A hypothalamic injection of desmethylimipramine, a blocker of NA re-uptake, potentiates feeding in starved rats

(Montgomery *et al.* 1971). This suggests that feeding is accompanied by NA release, and potentiation of eating can be facilitated by drugs that prolong the action of NA in the synaptic cleft by the stimulation of release and/or inhibition of re-uptake, or by direct agonists.

The stimulation of feeding by destruction of the noradrenergic innervation to the PVN is thought to be due to the destruction of NA efferent projections, rather than NA afferent innervation. It is thought that the NA acts as an enhancer of food intake, in the PVN, by the inhibition of a PVN controlled satiety signal (Leibowitz *et al.* 1981, McCabe *et al.* 1984). NA (40nmol into the PVN) potentiates feeding by increasing meal size (by 20%) and feeding rate, without affecting meal frequency (Leibowitz *et al.* 1984), and depletion of brain NA, using FLA-63, an inhibitor of NA synthesis, causes a reduction in meal size (Leibowitz 1986, Leibowitz and Shor-Posner 1986b). The PVN α_2-noradrenergic receptors modulate the consumption of carbohydrate (Booth 1968, Leibowitz 1984). Diet selection studies, in rats, between carbohydrate or protein shows that PVN administration of clonidine (α_2-adrenoreceptor agonist) and amitriptyline (a tricyclic antidepressant that inhibits NA re-uptake), and peripherally administered chlorimipramine, stimulate food intake preferentially for carbohydrate. NA produces similar results (Leibowitz *et al.* 1985). NA acts specifically in the potentiation of carbohydrate intake, with a suppression of fat and protein consumption. PVN damage produces the same effect as NA stimulation of the PVN (stimulation carbohydrate intake, with the inhibition of protein intake), (Shor-Posner 1985), as PVN damage is thought to damage PVN efferent projections and not NA afferents to the nucleus (the so-called "satiety system"), (Hoebel and Leibowitz 1981). Tricyclic anti-depressants and clonidine treatment in humans, and rats, causes weight gain (Leibowitz *et al.* 1978a/b). This is thought to be due to their potent action on NA release (in the PVN), (Leibowitz 1978b). Further evidence for the role of NA in carbohydrate nutrition comes from the close association of hypothalamic NA activity with corticosterone and insulin (both of which have important impacts on glucose metabolism).

Glucocorticoid status correlates to hypothalamic NA activity. Situations of increased energy expenditure i.e. food deprivation and stress, cause an increase in NA turnover in

the PVN (Leibowitz and Shor-Posner 1986), and a site specific down regulation of PVN α_2-noradrenergic receptors (Jhanwar-Uniyal *et al.* 1986a/b, Jhanwar-Uniyal and Leibowitz 1986). The initiation of the feeding process is thought to be via the PVN NA system (Jhanwar-Uniyal *et al.* 1986a/b, Leibowitz 1985, Levine *et al.* 1983, Bhakthavatsalam and Leibowitz 1985). The α_2-NA receptor feeding response of the PVN is dependent on an intact hypothalamic-pituitary-adrenal axis (Leibowitz and Shor-Posner 1986). Plasma corticosterone, in a dose-dependent manner up-regulates the number of α_2-NA receptors in the PVN (Ixart *et al.* 1982, Jhanwar-Uniyal *et al.* 1986, Leibowitz 1985). Adrenalectomy abolishes PVN NA-induced hyperphagia, and is restored with corticosterone replacement therapy (Leibowitz 1986, El-Rafai and Chan 1986a).

GABA

GABA acts on the PVN to stimulate food intake (Cooper 1983, Grandison and Guidotti 1977, Kelly and Grossman 1979, Kimura and Kiriyama 1975). Injection of muscimol (GABA agonist) and benzodiazepines into the PVN cause a stimulation of feeding which can be reversed by GABA receptor antagonism. GABA receptor antagonists also inhibit NA-induced feeding by co-injection into the PVN.

Neuropeptide Y

Neuropeptide Y (NPY), a pancreatic polypeptide, is highly concentrated in the PVN region of the hypothalamus, and potently stimulates food intake activity, insulin secretion and body weight gain, and reduces BAT activity (Clark *et al.* 1984, Levine and Morley 1984, Stanley and Leibowitz 1984). NPY is found to co-exist, in afferent neurones to the PVN, with NA and ADR (Everitt *et al.* 1984). The effect of NPY to increase feeding may not be mediated by NA, as blockade of α-ADR receptors does not prevent NPY-stimulation of feeding, whilst abolishing the NA effect (Stanley and Leibowitz 1985). Food deprivation increases PVN levels and release of NPY. Several classes of NPY receptors have been classified, called Y1, Y2, Y3 and Y4 subtypes (Michel 1991). A NPY receptor, the Y5 (NPY "feeding" receptor) has recently been isolated, cloned and characterised (Michel 1991, Gerald *et al.* 1996).

Opioids

Dynorphin and β-endorphin enhancement of feeding response, through μ-, δ- and κ-receptors (Leibowitz and Hor 1982, Morley *et al.* 1983, Woods and Leibowitz 1985, Yim and Lowry 1984) occurs in the PVN, VMH, PFN, amygdala and globus pallidus. Opioid peptide induced feeding is abolished by α-ADR receptor blockade (Leibowitz and Hor 1982, Tepperman *et al.* 1981). Morphine-induced eating stimulates NA turnover and is attenuated by adrenalectomy and restored by corticosterone replacement therapy (Bhakthavatsalam and Leibowitz 1986). Opioids and NA both inhibit the firing rate of PVN neurones (Moss *et al.* 1972, Pittman *et al.* 1980). These findings suggest a possible interaction of opioids and NA in the PVN region in affecting satiety. Central administration of a κ-receptor agonist (U50488) attenuates inhibition of a high-fat diet intake induced by enterostatin (Barton *et al* 1995) and alone stimulates high-fat diet intake in sated rats. Galanin similarly induces high-fat consumption in sated rats (Barton *et al.* 1995a, 1996), which is reduced by naloxone (Barton 1995b), μ-receptor antagonism but not κ-receptor antagonism. K-receptor antagonism, on the other hand, reduces high-fat intake in fasted (but not sated) rats. No effect of μ-receptor antagonism was seen in fasted rats. It is postulated that there is a common opioid pathway for the modulation of fat intake with galanin-induced feeding of high fat modulated by a μ-receptor pathway, with feeding induced by fasting modulated by a κ-receptor pathway (Barton *et al.* 1995b).

The role of different opioids in affecting feeding by differing mechanisms is exhibited by the observation that the attenuation of opioid hyperphagia by adrenalectomy only occurs with μ-agonists and the δ- and κ-agonists failure to respond to adrenalectomy suggests another corticosterone-independent hypothalamic stimulation of feeding (with or without NA). Lesions of hypothalamic NA, with 6-OH DA, do not affect morphine-induced feeding, suggesting another NA-independent mechanism.

1.3.2 Inhibitory neurotransmitters.

Dopamine/noradrenaline/adrenaline

Dopamine (DA), and to a lesser extent noradrenaline (NA) and adrenaline (ADR), exert an inhibitory action on feeding behaviour (Leibowitz and Brown 1980b). Opinion is that the site of action for the catecholaminergic inhibition of food intake is the perifornical region of the hypothalamus, the lateral hypothalamus, (PFN or LH), (Leibowitz and Brown 1980b). The action of DA in this site contrasts to the dopaminergic activity in other brain areas, where it generally exerts a stimulatory action on motivated behaviour, primarily in the striatum, which includes feeding (Leibowitz 1986). The PFN is richly innervated with dopaminergic terminals and receptors (Leibowitz and Shor-Posner 1986). Starved rats have a reduced intake, compared to control rats, after injection of DA into the PFN, with chronic treatment causing a pronounced anorexia.

Electrical stimulation and electrolytic lesions of the PVN have opposing effects in promoting and inhibiting feeding respectively (Leibowitz and Shor-Posner 1986). The role of catecholamines in the modulation of feeding behaviour has been greatly elucidated by the use of D-amphetamine (AMP) in feeding studies, and the manipulations of the anorexia induced by AMP treatment. AMP stimulates hypothalamic DA neurones and also inhibits feeding when injected into the PFN (Leibowitz and Shor-Posner 1986). DA turnover is increased following AMP administration peripherally (Leibowitz and Shor-Posner 1986). DA antagonists (haloperidol and chlorpromazine) stimulate feeding also (Leibowitz and Shor-Posner 1986). The receptors involved are dopaminergic and β-adrenergic (Leibowitz and Shor-Posner 1986). The innervation of the PVN may originate from the dorsal pons, possibly the locus coeruleus and subcoeruleus. The efferent projections follow a periventricular route caudally to the dorsal vagal complex (Sawchenko *et al.* 1983, Weiss and Leibowitz 1986, McCabe *et al.* 1984). The innervation of the PVN may originate in the A_1, A_2 or A_7 groups of cells, as damage to the ventral central tegmental tract blocks inhibition of feeding by β_2 or DA activation of the PFN. The DA contribution to this fibre system may originate from the A_8 or A_9 cells of the midbrain.

Tritiated AMP binds densely in the hypothalamus and also stimulates NA neurones, as well as DA neurones, in the hypothalamus. Lateral hypothalamic injection of ADR produces similar effects as DA, via a β-adrenergic receptor, and operates in close association with hypothalamic DA receptors (Leibowitz and Shor-Posner 1986). Injection of 150ng adrenaline into the PVN suppresses food intake with a latency of 1-2 minutes (Leibowitz and Rossakis 1978), by a β_2 receptor mechanism as the effect can be repeated using the β_2 agonist salbutamol and blocked by the β_2 antagonist butoxamine (Leibowitz and Brown 1980). The decrease in food intake following AMP treatment (peripheral and direct administration into the PFN) is by a selective decrease in protein consumption. DA receptor blockade in the PFN, by peripheral or local neuroleptic treatment, causes an opposite effect i.e. increasing food intake and weight gain (Leibowitz 1985, Leibowitz *et al.* 1981).

The lateral hypothalamic (perifornical) dopaminergic and β_2-adrenergic inhibitory zone, and the α_2-noradrenergic feeding facilitatory region of the medial (paraventricular) hypothalamus both co-ordinate in the control of meal pattern by their opposing effects on food intake and their specific individual effects on protein and carbohydrate intake respectively, which are regulated together with energy intake (Li and Anderson 1983, Leibowitz 1985).

Peptides

Peptides, administered centrally, which show inhibition of food intake include cholecystokinin (CCK), bombesin, neurotensin, CRF, vasopressin, anorectin, insulin, calcitonin, somatostatin, thyrotropin-releasing hormone, substance P and satietin (Morley *et al.* 1985, Leibowitz 1985, Smith and Gibbs 1987). The hypothalamic region is the most sensitive to these peptides in inducing a satiety effect, but the pons-medulla and amygdala also show satiety associated with these peptides (Leibowitz and Shor-Posner 1986, Leibowitz 1985). VMH or III ventricular infusion of these peptides produces hypophagia (Leibowitz and Shor-Posner 1986, Leibowitz 1985).

Food deprivation reduces hypothalamic CCK levels, which are restored following feeding (Baile and Della-Fera 1984). Neurotensin, calcitonin, CCK and CRF are

thought to act with NA in the PVN region of the hypothalamus (Faris *et al.* 1983, Dorfman *et al.* 1984, Van Housten *et al.* 1982, Freed and de Beaurepaine 1984, Morley *et al.* 1985), whilst the opioids interact with the catecholaminergic system of the PFN (Leibowitz 1986). Peripheral injections of CCK abolish feeding induced by PVN injections of NA (Myers and McCaleb 1981). See section 1.1.2.2. on the role of CRF in nutrient balance.

The modulation of feeding by these peptides is not fully understood but, co-existence with monoamines in hypothalamic neurones (Leibowitz 1985, Morley *et al.* 1985), suggests a possible interaction with the previous catecholaminergic systems mentioned.

5-HT (5-hydroxytryptamine/serotonin)

Hypothalamic 5-HT exerts an inhibitory effect on food intake (Blundell 1984, Blundell 1986, Li and Anderson 1983, Stepheson 1990, Blundell and Latham 1978, Cooper 1989, Garattini *et al.* 1988, Nathan and Rolland 1987, Silverstone and Goodall 1986b). Feeding produces an increase in extracellular 5-HT in the LH, as measured by microdialysis (Schwartz *et al.* 1989). Generally, the serotoninergic inhibition of feeding is modulated in the PVN (medial region) of the hypothalamus (and may be opposing the NA effect on the PVN) to produce satiety primarily for carbohydrate, with the sparing of protein intake (Blundell 1984).

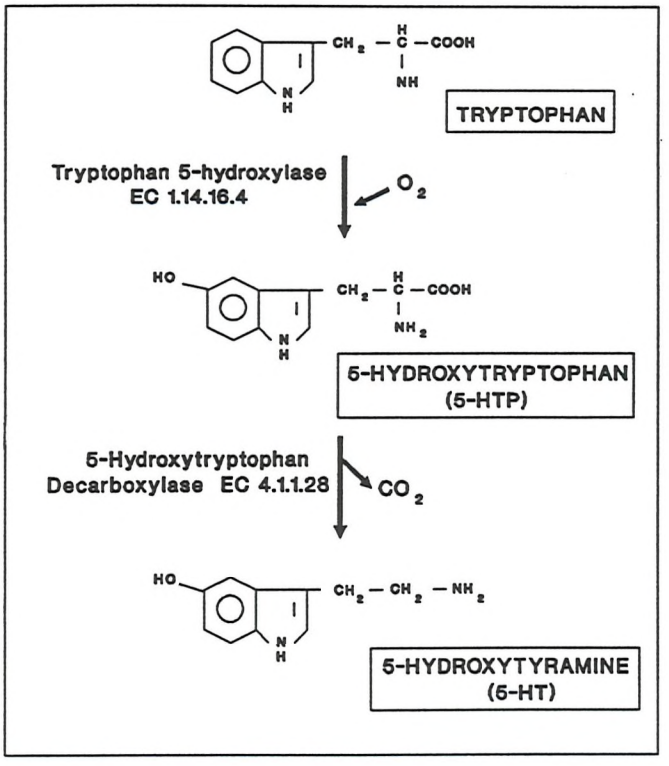
The hypothalamic region, in mammals, contains the highest concentration of 5-HT and 5-HT receptors (Pazos and Palacious 1985, Sawchenko *et al.* 1983). Administration of 5-HT into the hypothalamus, particularly into the PVN, reduces food intake (Blundell 1984). Leibowitz and Shor-Posner (1986) demonstrated that the inhibition of food intake by 5-HT administration to the PVN is dose-dependent. 5-HT injection into the PVN reduces size, duration and eating rate of meals, without affecting meal frequency (Rowland and Carlton 1986a). Fenfluramine (see section 1.4.) produces the same effects, which is known to act by increasing central 5-HT synaptic activity.

The PVN noradrenergic and serotoninergic system act antagonistically in feeding behaviour, as shown by NA PVN elicited eating being abolished by 5-HT (or

tryptophan) injections into the PVN (Leibowitz 1978a, Leibowitz *et al.* 1983, Weiss *et al.* 1986). The immediate precursor of 5-HT, 5-hydroxytryptophan (5-HTP), injected into the PVN also inhibits NA induced eating (Rowland and Carlton 1986b). The mechanism for 5-HTP inhibition of feeding is via 5-HT and not by direct receptor stimulation, as injections of RO 4-4602 and MK-468, which inhibit the 5-hydroxytryptophan decarboxylase enzyme, abolish 5-HTP feeding, and not 5-HT-induced feeding suppression (Rowland and Carlton 1986a/b). Peripheral 5-HT administration, at a dose that does not affect locomotor activity, sensorimotor faculties or conditioned taste aversion, produces a dose dependent reduction in food intake (Fletcher and Burton 1984, Fletcher and Burton 1985, Pollock and Rowland 1981). This peripheral effect acts by delaying gastric emptying, and not by central stimulation, as peripherally administrated 5-HT does not cross the blood brain barrier (Oldendorf 1971).

Central 5-HT synthesis, see figure 1.1, is dependent upon the levels of its precursor, tryptophan, centrally (see section 1.2.1.4). Tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan 5-hydroxylase [L-tryptophan, tetrahydropteridine-oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4], which is converted in to 5-hydroxytryptamine (5-HT) by the enzyme 5-hydroxytryptophan decarboxylase [aromatic L-amino acid carboxylase, EC 4.1.1.28]. Oxidative metabolism of 5-HT to 5-hydroxyindoleacetic acid (5-HIAA) represents the major pathway, in mammals, for inactivation of 5-HT. The overall conversion of 5-HT to 5-HIAA consists of a two step process (see figure 1.2). The first step is the oxidative deamination of 5-HT to 5-hydroxindoleacetaldehyde catalysed by monoamine oxidase [amine:oxygen oxidoreductase (deaminating), EC 1.4.3.4]. The second step involves dehydrogenation of the intermediate aldehyde by aldehyde dehydrogenase [aldehyde NAD^+ oxidoreductase, EC 1.2.1.3] to yield 5-hydroxyindoleacetic acid, with lesser conversion of 5-hydroxyindoleacetaldehyde by alcohol dehydrogenase [alcohol NAD^+ oxidoreductase, EC 1.1.1.1] to 5-hydroxytryptophol.

Figure 1.1. Synthesis of 5-HT.

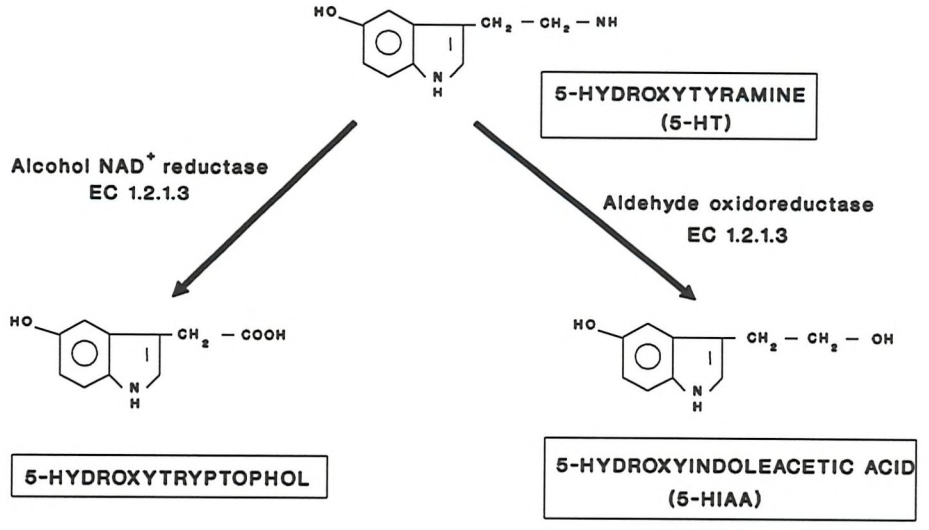


The 5-HIAA, diffuses from the brain (after synaptic release) into the cerebrospinal fluid (CSF), and is then eliminated by an anion transport mechanism of the choroid plexus (Fuller 1980a, Miyamoto *et al.* 1990). The transport can be blocked with probenecid, with the subsequent 5-HIAA accumulation in the CSF giving an indication of central 5-HT turnover (Fuller 1980a).

5-HT modulates the intake of carbohydrate, with increasing 5-HT activity resulting in a reduction in the proportion of carbohydrate in the diet (Shor-Posner *et al.* 1987). 5-HT injection into the PVN region of the hypothalamus reduces carbohydrate intake, with the sparing of protein intake (Leibowitz and Shor-Posner 1986a).

5-HT antagonism, with peripherally administered cyproheptadine, has the opposite effect, in that carbohydrate (and fat) intake is stimulated, whilst protein is not affected (Shor-Posner *et al.* 1986). Human studies show that the increase in 5-HT activity inhibits the consumption of carbohydrate (reduced craving for carbohydrate rich snacks), (Silverstone and Goodhall 1986, Blundell and Rogers 1980, Wurtman and Wurtman 1986).

Figure 1.2. Inactivation of 5-HT.



Multiple 5-HT receptor subtypes have been isolated, and are divided into seven main groups (at the time of writing), the 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7 subtypes. The 5-HT_1 groups are further subdivided into 5-HT_{1A}, 5-HT_{1B} (previous 5-$HT_{1D\beta}$), 5-HT_{1D} (specifically 5-$HT_{1D\alpha}$), 5-HT_{1E} and 5-HT_{1F} (previous 5-$HT_{1E\beta}$/5-HT_6) receptor types (Peroutka *et al.* 1981, Peroutka and Synder 1983, Glennon 1987, Frazer *et al.* 1990, TIPS supplement 1996, Hartig *et al.* 1996, Hoyer *et al.* 1994, Peroutka 1995). 5-HT_{1A} receptors are located pre-synaptically (autoreceptors), with the 5-HT_{1B} receptors located post-synaptically (Pedigo 1981, Peroutka 1984). DNA sequencing of the receptor types show that the receptor formerly known as the 5-HT_{1C} receptor is more closely related to 5-HT_2 receptors than the other 5-HT_1 types, as both are activated by a phosphinositide second messenger system (Hartig 1989, Hoyer 1988). The 5-HT_{1C} receptor has now been re-classified as 5-HT_{2C} (TIPS Supplement 1996). The secondary messenger system of the other 5-HT_1 receptors involves inhibition of adenylyl cyclase (Schoeffter *et al.* 1988), although the actions of the 5-HT_{1E} receptors are thought to G protein coupled. 5-HT_2 groups are further subdivided into 5-HT_{2A} (previous D-receptor/5-HT_2), 5-HT_{2B} (previous 5-HT_{2F}) and 5-HT_{2C} (previous 5-HT_{1C}) receptor types (Baxter *et al.* 1995). The 5-HT_3 (previous M-receptor) receptor is unique among the seven 5-HT receptor classes in that it

functions not as a G-protein coupled receptor but as a direct membrane spanning cation channel-gated receptor (Derkach 1989, TIPS Supplement 1996). The 5-HT_4 receptors stimulate adenylyl cyclase (Dumuis *et al.* 1988, Clarke 1989). The 5-HT receptor is divided into 5-HT_{5A} (previous 5-$HT_{5\alpha}$) and 5-HT_{5B} (previous 5-$HT_{5\beta\alpha}$) subtypes whose receptor secondary messenger system has yet to be classified. The 5-HT_6 and 5-HT_7 (previous 5-HT_x) receptors are G protein linked (TIPS Supplement 1996).

The action of 5-HT in the modulation of hypothalamic eating behaviour is thought to be mediated via 5-HT_1 receptors. The 5-HT mediation of feeding acts via 5-HT_{1B} receptors, and the 5-HT_{1A} receptors may be the mediator of hyperphagia (Dourish *et al.* 1986, Bendotti and Samanin 1987). Evidence for these statements is demonstrated by the 5-HT-induced suppression of feeding in the PVN not being affected by the 5-HT_2 receptor antagonist ritanserin (Massi and Marini 1987), whilst the antagonism to 5-HT_{1A} and 5-HT_{1B} receptors using metergoline, antagonises this effect. The anorexia induced by quipazine, a 5-HT_{1B} receptor agonist can be antagonised by metergoline (Samanin *et al.* 1977, Samanin *et al.* 1978, Blundell 1984, Dourish *et al.* 1986, Bendotti and Samanin 1987, Hewson 1988), which further suggests a 5-HT_{1B} receptor mediation of 5-HT anorexia.

Further support for 5-HT mediation of food intake modulation by the PVN region of the hypothalamus, in rats, is demonstrated by the presence of a dense population of 5-HT_{1B} receptors in this region. In contrast to the 5-HT_{1B} and the 5-HT_{1D} receptors, the 5-HT_{1A}, 5-HT_{2C} (previous 5-HT_{1C}) and 5-HT_{1E} subtypes exist in all mammalian species examined. The human 5-$HT_{1D\beta}$ and the rat 5-HT_{1B} are classified together as 5-HT_{1B}; the 5-HT_{1D} appellation refers specifically to the 5-$HT_{1D\alpha}$ receptor. The rat 5-HT_{1B} receptor exhibits a unique pharmacology (Oksenberg *et al.* 1992, Hartig *et al.* 1996). The 5-HT_{1B} receptor is not found in the human, but is similar to the human 5-HT_{1D} receptor.

5-HT_{1B} receptor agonists, RU-24969, quipazine and *m*-chloro-phenylpiperazine (m-CPP), induce anorexia whilst the 5-HT_{1A} agonist, 8-hydroxy-2(di-*n*-propylamino) tetralin (8-OH DPAT) causes hyperphagia by the inhibition of 5-HT release from the pre-synapse (Arvidsson *et al.* 1981, Bendotti and Samanin 1986, Chaouloff and

Jeanrenaud 1988, Dourish *et al.* 1986, Hjorth *et al.* 1982, Bendotti and Samanin 1987, Tricklebank *et al.* 1985). Section 1.1.1.1. dealt with the effect of insulin on the 5-HT activity. Following the consumption of a large carbohydrate meal at the beginning of the night cycle, in animals, an increase in the plasma insulin causes an increase in the amount of tryptophan available for uptake into the brain, and causes brain tryptophan and 5-HT levels to rise (Fernstrom 1985). Protein ingestion, in animals, decreases brain tryptophan uptake and therefore reduces CNS 5-HT activity (Fernstrom 1985). Humans exhibit similar effects (Fernstrom 1985, Fernstrom *et al.* 1979, Moller 1985). The convergence of evidence indicates that the α_2-noradrenergic and serotoninergic antagonism in the PVN mediates feeding behaviours between these two systems.

5-HT levels peak in the hypothalamus, during the early hours of the dark cycle (Agren *et al.* 1986, Faradji *et al.* 1983, Hery *et al.* 1972, King *et al.* 1985), i.e. inhibitory effect on carbohydrate intake at the beginning of the dark cycle, and PVN NA peaks similarly at the start of the dark cycle (Agren *et al.* 1986, Leibowitz et. al. 1988) to stimulate food intake during this time period, circadian variations of tyrosine hydroxylase, aminotransferase and tryptophan hydroxylase are also seen (Cahill and Ehret 1981).

It would appear that the hypothalamic NA and 5-HT component (with other factors) become specifically activated at the beginning of the nocturnal cycle (where the majority of feeding behaviour, in rats, occurs), to act antagonistically with each other in the control of the ratio of carbohydrate to protein ingestion. The modulation of this ratio, by the animal, maintains nutrient balance by affecting individual intake components.

The list of inhibitors of feeding behaviour indicated is not exhaustive. Other agents that are known to inhibit food intake include satietin, peptide (pyroGlu-His-Gly-OH), (-)-hydroxycitrate, threochlorocitrate, atropine, oestrogens, ceruletide, bombesin, enterogastrone, somatostatin, thyrotropin-releasing hormone, calcitonin, prostaglandins, arachadonate, linolenate, cocaine, naloxone, alcohol, aspartame and tetrahydrocannabinol (Blundell 1991).

5-HT is also closely involved in a wide variety of physiological processes such as nociception, cardiovascular function and thermoregulation, and behavioural/ environmental processes such as aggression, the modulation of the sleep-wake arousal cycle and stress behaviour, together with the previously reviewed modulation of feeding/nutrient selection.

The whole process of ingestive behaviour regulation must be inter-linked by hormonal and neural connections to integrate the ingestive feedbacks, satiety, specific hunger responses, bodily demands for the specific nutrients and energy and the mobilisation of nutrients from body stores.

1.4. Fenfluramine.

An important tool for the understanding of the role of one important modulator of feeding behaviour, 5-HT, has been the development of the drug fenfluramine, and more specifically the D(+)-isomer. Manipulations of feeding behaviour, and antagonism to the actions of the drug, enable the 5-HT component of feeding behaviour to be isolated and also discover interactions with other feeding behaviour signals.

Fenfluramine (*N*-ethyl-α-methyl-*m*-trifluoromethyl-phenylethylamine), is a potent appetite suppressant drug, similar in structure to amphetamine (see figures 1.3 and 1.4), but possesses a sedative and tranquillising activity with low abuse potential, rather than the psychomotor stimulatory effect and high abuse potential associated with amphetamine. Fenfluramine was first presented by Laszlo Beregi *et al.* (1963), from the Science-Union et cie Société Française de Recherche Médicale, Patent application No.FR1, 3244.220 (1960), Beregi and Hugon.

The structure of Fenfluramine reveals a chiral centre at the α-carbon atom, producing two optical isomers, the D-(+) S-configuration and the L(-)- R configuration isomers of fenfluramine.

Clinically, fenfluramine is prescribed as its hydrochloride salt (CAS 404-82-0, 768S0, molecular weight 267.7). Preparations of fenfluramine are marketed worldwide under the brand names Ponderax® (20mg and 40mg tablets), Precaps® (sustained release capsules), Ponderal®, Pondimin®, Ponderex®, Grazilan®, Minifage®, Miniphage®, Ponflural® and Ganal®. The slow release capsules are more effective than single bolus dosage tablet forms (British National Formulary (4) 1982 4.5.1 and 4.5.2; ABPI, Data Sheet Compendium 1985-1986). As will be discussed later, the D-(+) isomer of fenfluramine (D-Fen) exhibits a greater anorectic activity, (Rowland and Carlton 1988), this drug is also available clinically (Dexfenfluramine, Adifax®, DF®, Isomeride®).

Typical dose regimes of approximately 0.3-0.4mg kg^{-1} day^{-1} are used, depending on the severity of the obesity, with a recommended daily dose of 30mg day^{-1} (Finer *et al.* 1985).

Figure 1.3. Structure of Amphetamine

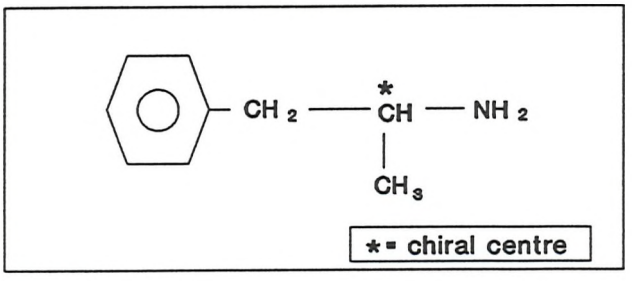
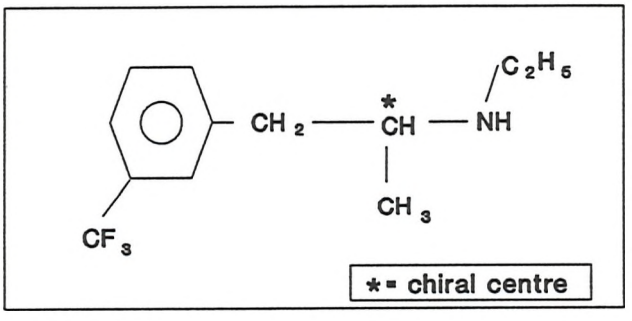


Figure 1.4. Structure of Fenfluramine



1.4.1. Fenfluramine Pharmacokinetics.

Oral doses (1mg kg^{-1}) of D-Fen are well absorbed, in all animals, with peak plasma levels of 30-60ng ml^{-1} seen after 1 hour for rodents, and 2 to 4 hours in dogs, monkeys and humans (Campbell 1991). Almost all the dose is excreted into the urine within 3 to 5 days, with less than 5% lost in faeces (Campbell 1991).

Fenfluramine is highly lipid soluble with an octanol buffer partition coefficient of 8.8, and a chloroform/water (pH 7.4) coefficient of 32, and has plasma albumin binding of between 30 to 40% (Campbell *et al.* 1991). Because of this high lipid solubility and low albumin binding, fenfluramine is highly sequestrated into adipose tissue, muscle and brain tissue (Beckett and Salmon 1972, Campbell 1973, Caccia *et al.* 1982, Bizzi *et*

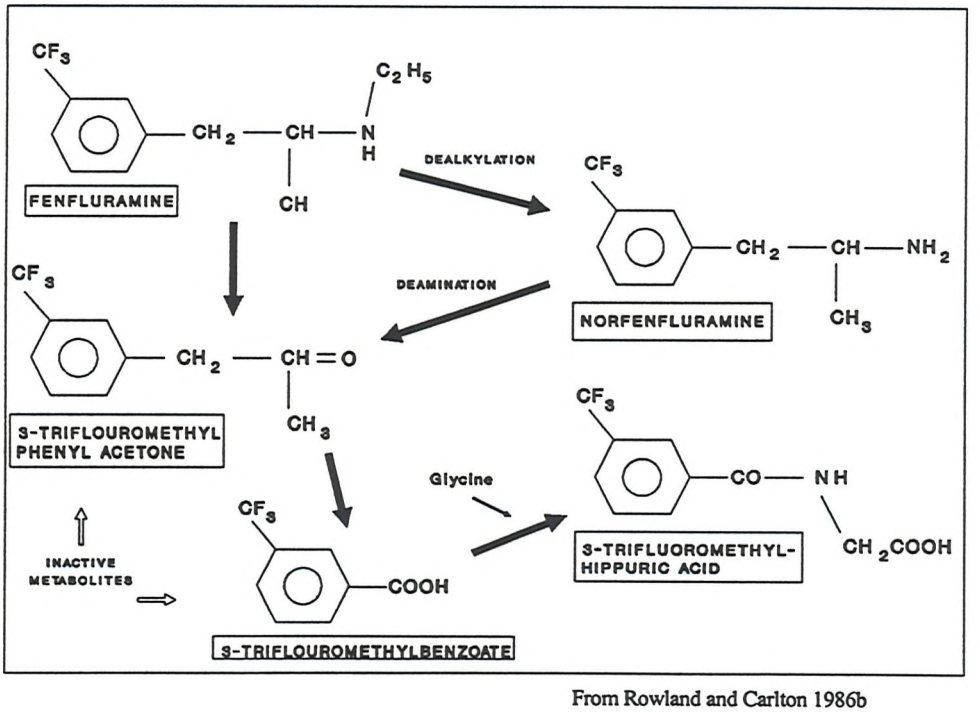
al. 1978), with a volume of distribution of 20 to 40 litres kg^{-1}. D-Fen accumulates in 5-HT containing brain regions (hypothalamus, striatum and cortex, with lower levels in the brainstem, hippocampus and cerebellum) at levels 30 to 40 times plasma concentration. Adipose tissue sequestration yields similar concentrations (1nmole/g), in both lean and obese gold-thioglucose treated mice (Bizzi *et al.* 1978). This sequestration is approximately 20% brain sequestration levels, but again still higher than blood levels. The adipose tissue in the obese mice sequester higher amounts of fenfluramine (by the presence of increased amounts of adipose tissue present), than compared to lean animals, and so act as a slow releasing pool of Fen, and therefore prolonging the duration of Fen's action. D-Fen doses, orally, of 2.5 and 10mg kg^{-1} (8 fold increase) results in an increase of brain deposition of 10 and 335µg $hour^{-1}$ respectively (a 33 fold increase), (Garattini *et al.* 1988). Brain D-Nor-Fen levels for the same animals were 34 and 262µg $hour^{-1}$ deposited respectively (a rise in proportion to the dosage of D-Fen).

Fenfluramine is metabolised principally by the liver by de-ethylation to form the Nor-Fenfluramine molecule (see figure 1.5). The anorectic potency of Nor-Fen is similar to that of fenfluramine, but has a plasma half-life many fold that of fenfluramine (Beckett and Salmon 1972, Campbell *et al.* 1979). Metabolism of Nor-Fen to 3-trifluromethyl-phenyl acetone and then to 3-trifluorobenzoic acid (both of which possess no anorectic activity), finalises in conjugation with glycine to produce 3-trifluorohippuric acid (Beckett and Salmon 1972), the major excretory product of Fen, which is excreted in the urine (Bruce and Maynard 1968). In rats, because the half-lives of both isomers of Nor-Fen (D-Nor-Fen and L-Nor-Fen) exceed those of their precursor drugs, D-Fen and L-Fen respectively, the plasma levels, particularly during chronic treatment, exceed that of the drug. The plasma half-life ($T_{½}$) of Fen varies from 1 to 2 hours in the guinea pig and rat, 4 hours in the mouse (greater in obese mice) and 12 to 20 hours in humans (Fuller *et al.* 1978, Caccia *et al.* 1982), with the half-life for Nor-Fen between 12-24 hours. In humans however, the concentration of Fen always exceeds that of the Nor-Fen molecule, despite the slightly higher plasma half-life of the latter ($T_{½}$ Nor-Fen = 24 hours), (Caccia et. al. 1982, Campbell *et al.* 1979, Garattini *et al.* 1979, Bizzi *et al.* 1978, Caccia *et al.* 1985, Caccia *et al.* 1981). In rats, therefore, the

major anorectic compound following fenfluramine treatment is the parent drug for only 2-6 hours after its administration, after which Nor-Fen predominates. In monkeys and dogs the metabolism of Fen is intermediate between that of human and the rat (Garattini *et al.* 1979, Garattini *et al.* 1986). Mice come closest to displaying a Fen/Nor-Fen profile similar to human, in that Fen levels exceed those of Nor-Fen at all times, even though the Fen plasma $T_{½}$ is only 4 hours (Bizzi *et al.* 1978, Caccia *et al.* 1982, Pawan 1970).

L-Fen is dealkylated into L-Nor-Fen faster than compared to the dealkylation of D-Fen into Nor-Fen. So, at various times of acute, and chronic, treatment with the raceamate, the levels of D-Fen may exceed those of L-Fen but L-Nor-Fen may exceed those of D-Nor-Fen (Rowland and Carlton 1986b, Morgan *et al.* 1972). This may have importance in the interpretation of results obtained from short-term administration of the raceamate of Fen compared to administration of individual isomeric preparations alone.

Figure 1.5. Metabolism of Fenfluramine.



From Rowland and Carlton 1986b

Side effects of fenfluramine treatment increase at plasma levels of 120ng ml^{-1} and greater, and include symptoms such as nausea, diarrhoea, headache, dizziness, sedation, dry mouth, flatulence, abdominal discomfort, constipation, fatigue, reduced or

lightening of sleep, increased depression, nightmares, palpitations, shivering, fluid retention, increased urinary frequency, impotence, loss of libido, skin rash, alopecia and haemolytis anaemia. Overdosage of Fen results in vomiting, mydriasis and nystagmus, tremours, rigidity, hyperpyrexia, sweating, hyper-reflexia, facial flushing, hypertension, cardiac arrythmias, convulsions, unconsciousness and death from cardiac arrest (Fleisher and Campbell 1969, Gold *et al.* 1969). Many of the symptoms are typical of the effects of d-amphetamine over-dosage. Fen overdosage is treated by stomach pumping (to remove any remaining unabsorbed GI tract Fen), anticonvulsant treatment, chloropromazine or β-adrenoceptor blocking drugs, artificial respiration, artificially induced increase in urinary excretion by forced acid diuresis and continual cardiac monitoring.

1.4.2. Fenfluramine and body weight changes.

Plasma levels greater than 1μM produce weight losses of approximately 0.5-1 kg $week^{-1}$ in obese humans (Innes *et al.* 1977, Rowland 1986). The associated weight loss is not sustained, and usually patients maintain a stable lowered body weight approximately 10 kg below that of the initial weight (Douglas *et al.* 1983, Innes *et al.* 1977, Stunkard 1981, Finer *et al.* 1985, 1988, Jori *et al.* 1978). Discontinuation of drug therapy, without a rigid diet control results in reversal of this weight loss (Douglas *et al.* 1983, Stunkard 1981). The recovery of weight loss in humans and animals may be a reflection of a metabolic adaptation to a lowered body mass (lowered body set point) rather than a tolerance to the anorectic activity of Fenfluramine.

Rats, treated with Fen and D-Fen, display similar proportionate weight losses as in humans, with the initial weight loss followed by a weight gain (if in growth phase) or the maintenance of a body weight below that of its saline control animals (Duhault *et al.* 1979, Brindley 1985, Fantino *et al.* 1986, 1988). The ED_{50}, in rats is 1.3mg kg^{-1} (Garattini *et al.* 1987). Fenfluramine produces greater weight loss effects in obese rats. Medial hypothalamic obesity is reduced by Fen treatment, compared to saline control mice and rats (Abdallah 1968, Bernier *et al.* 1969, Duahault *et al.* 1979). Cafeteria fed rats treated with Fen fail to become obese, or reverses the induced obesity (Kirby *et al.*

1978, Yudkin and Miller 1971). Fenfluramine attenuates weight gain in ovariectomised rats, whilst saline treated ovariectomised rats become obese (Souquet and Rowland 1990). The sequestration of Fen by adipose tissue may increase the bioavailability, in the obese models discussed here, by its slower sustained release maintaining a plasma level of Fen longer than their lean counterparts.

1.4.3. Fenfluramine and food intake.

Chronic treatment with Fen causes the initial fall in food intake, but returns to control values after 3-7 days (Even and Nicolaidis 1986, Brindley *et al.* 1985). D-Fen, as 5-HT, reduces the proportion of carbohydrate in the diet (Leibowitz and Shor-Posner 1986, Burton *et al.* 1981). Meal size is reduced in pre-fasted rats given Fen (Blundell 1986), and in non-food deprived rats (Moses and Wurtman 1984). Following a single injection of Fen, there is no catch up of food intake in the latter half of a 24 hour feeding schedule, when the plasma and brain levels of Fen will be expected to be considerably low (Rowland and Carlton 1986b). D-Fenfluramine (D-Fen) and D-Nor-Fenfluramine (D-Nor-Fen) cause a similar effect to 5-HT, in reducing carbohydrate intake with a sparing of protein intake (Blundell and McArthur 1979, McArthur and Blundell 1983, Hirsch *et al.* 1982, Wurtman and Wurtman 1977, Wurtman and Wurtman 1979). D-Fen selectively decreases carbohydrate intake in the early dark period (ED_{50} 0.1mg kg^{-1}), with a decreased potency in reducing food intake in rats starved for 24 hours (ED_{50} 1.3mg kg^{-1}), (Campbell 1991). Burton *et al.* 1981 demonstrated that nocturnal feeding is inhibited following p.m. injection, and the next day (light period) food intake does not exceed the normal low food intake, associated with the animals during the light period, of saline treated rats. The nocturnal feeding is not affected by either a.m. or p.m. injections. Rowland and Carlton (1986) show that a reduction in body weight and food intake occurs when given daily i.p. injections of Fen at a.m. or p.m. times. Trials with rats, and humans, demonstrate that the reduction of energy intake is the suppression of carbohydrate intake with a sparing of the protein component, via a serotoninergic system. This specific reduction of carbohydrate intake occurs only at low doses of D-Fen, whilst higher doses of D-Fen non-selectively reduced food intake (Hirsch *et al.* 1982).

1.4.4. Peripheral effects of Fenfluramine.

Fen and D-Fen inhibit gastric emptying (Davies *et al.* 1983, Rowland and Carlton 1984, Booth *et al.* 1986, Rowland and Carlton 1986). Stomach, intestinal, duodenum or caecum levels of 5-HT are however not depleted with Fen treatment (Duhault and Verdavainne 1967, Costa *et al.* 1971, Rowland and Carlton 1986). Rats in which gastric accumulation is prevented by a fistula should not display anorexia following Fen treatment if the effect of the drug is mediated by a gastric inhibitory effect. However, Rowland and Carlton (1986) demonstrated that gastric fistulated rats display the typical response to Fen, and this indicates that the anorectic effect of Fen is not mediated via a gastric mechanism.

Fen (and D-Fen) is found to lower plasma glucose (Kneebone 1971, Bliss 1972, Andersen *et al.* 1993). Diabetic humans, mice and rats show impaired glucose tolerance and insulin secretion (Dykes 1973), which is improved following fenfluramine treatment. The glucose effect is not a result of the increase in insulin release (Pinder *et al.* 1975, Cerrato *et al.* 1978). Muscle is the major peripheral tissue involved in glucose homeostasis. Insulin dependent glucose uptake by peripheral tissues is stimulated by Fen (Kirby and Turner 1975, Pinder *et al.* 1975), although Sasson *et al.* (1989, 1990) report no change in basal or insulin-stimulated glucose uptake in skeletal muscle of rats by Fen. The mechanism of action is not understood, but studies have shown that Fen may bind directly to insulin receptors (Harrison *et al.* 1975), or affect insulin sensitivity (Verdy *et al.* 1983). Some effects of Fen on glucose homeostasis are mediated through hepatic interactions. Fen inhibits gluconeogenesis and fatty acid synthesis, and several microsomal enzymes. Fen stimulated glucose uptake is 5-HT mediated as it is inhibited by methysergide and is stimulated by the addition of 5-HT or 5-HTP, in *in vitro* tissues (Turner and Kirby 1978).

Obese and non-obese humans, treated with Fen, display increased plasma levels of free fatty acids, glycerol, ketone bodies, and decreased levels of triglycerides and total plasma lipids (Pawan 1970). Fen, in animals, reduces the activity of the intestinal triglyceride synthesising enzyme, monoacylglycerol acyltransferase, which catalyses

the formation of triglycerides from monoglycerides in the intestinal lumen (Dannenberg *et al.* 1973), and also decreases fat absorption from the diet (Bizzi *et al.* 1973, Garattini *et al.* 1975).

The peripheral actions of Fen, in affecting glucose and lipid metabolism, play a role in producing the anorexia of D-Fen, but the primary anorexic action of Fen is, as will be shown later, mediated centrally. Indirect evidence for this is derived from systemic injection studies. Centrally acting, as opposed to peripherally acting, 5-HT receptor blockers antagonise the decrease in food intake by Fen. Metergoline, a central 5-HT antagonist, but not xylamide, a 5-HT antagonist with limited blood brain barrier diffusion ability, counteracts the reduction of oral sucrose solutions by Fen (Borsini *et al.* 1985). Peripheral 5-HT_2 receptor antagonism with xylamide does not block D-Fen effects on gut motility (Samanin *et al.* 1991) or food intake (Borsini *et al.* 1982, Neill and Cooper 1989), suggesting that any anorexic effect of D-Fen dependent on gut motility are mediated centrally. The peripheral effects of D-Fen (and D-Nor-Fen), compared to centrally mediated effects, can be largely discounted as the usual brain:serum ratio of D-Fen (and D-Nor-Fen) are >30-70:1.

1.4.5. Fenfluramine and the hypothalamic-pituitary-adrenal (HPA) axis.

Evidence suggests that Fen produces stimulation of the HPA. Dose-dependent increases in plasma corticosterone occur following Fen treatment (Fuller *et al.* 1981, McElroy *et al.* 1984, Barbieri 1984). Fenfluramine stimulates CRF and ACTH secretion from the hypothalamic slices and is antagonised by methylsergide (Holmes *et al.* 1982). An acute dose of D-Fen causes secretion of CRF, ACTH and corticosterone, in rats (De Souza *et al.* 1989, Serri and Rasio 1987). The stimulation of corticosterone secretion by D-Fen is dependent on an intact central 5-HT system as electrocoagulation of the raphe and 5,7-DHT block the response (Schetti *et al.* 1979). Chronic D-Fen treatment, to rats, however has been shown to decrease the release of corticosteroids (and fatty acids) in response to stress (Brindley 1988). Stimulation of corticosteroid release, in conscious rats, is abolished by selective 5-HT_{2A} receptor antagonism (Barbieri *et al.* 1984), which does not affect corticosteroid secretion alone.

The exact role of fenfluramine in the function of the (HPA) axis is not known, and specifically the CRF effects may have important implications in the understanding of the modulation of feeding.

Rowland and Carlton (1986b) dismiss any importance of *"the anti-obesity effects of fenfluramine being related to the hypothalamic-pituitary-adrenal axis, as the metabolic effects of glucocorticoids are the reverse of the effect of fenfluramine"*. This may be true, in terms of the glucocorticoid effects, as glucocorticoids and fenfluramine have opposing effects on such factors as insulin and glucose secretion, but the significance of CRF itself (which is not fully understood) may be important. See section 1.1.2.2. for 5-HT and CRF review.

Arase *et al.* (1989c) demonstrated that Fenfluramine (racemate) anorexia is potentiated by adrenalectomy. Adrenalectomy, in Fen treated animals, causes a cessation of the recovery to Fen anorexia, (for the study period) and prolonged hypophagia (observed in sham Fen-treated animals) and an increase in the sympathetic nervous system activity compared to either adrenalectomised or fenfluramine treated animals alone, as indicated by the binding of [^{3}H]GDP to BAT. The interaction of adrenalectomy (removal of endogenous corticosterone) and Fen, in the potentiation of body weight loss by hypophagia and increased activity of the SNS activity, is postulated to be via an interaction between central CRF and Fen. Adrenalectomy increases central CRF concentration, particularly in the PVN, and CRF is a potent inhibitor of food intake, and a sympathetic activator (see section 1.1.2.2.). Fenfluramine stimulates the release of CRF centrally (the importance of which is not known), and the interaction between Fen and CRF may produce the potentiation of Fen anorexia by an increased stimulation of CRF release.

Rowland and Dotson (1993) report *"(data)...do not support the idea of an interactive role of glucocorticoids and brain serotonin in anorexia"*. They observed that the increased anorexia and body weight reduction observed in adrenalectomised D-Fen treated animals, compared to saline controls was accompanied by a loss of appetite for NaCl and postulate that the rapid detoriation of the animals may have been due to

excessive loss of sodium ions and extracellular volume (although they did not assess this directly).

1.4.6. Metabolic effects of Fenfluramine.

The observation that body weight is maintained at a lower level following D-Fen treatment, even though food intake is restored to control levels, prompts suggestions that D-Fen (and Fen) is causing an additional factor to produce weight loss other than the hypophagia. A route for a reduction in body weight is an increase in the metabolic rate, the transfer away from storage mechanisms or a reduction in food utilisation of the animals. No significant changes in metabolic rate, in humans or animals, have been demonstrated using Fen (Yudkin and Miller 1971, Rothwell *et al* 1981). Other studies, however, have demonstrated an increase in the oxygen consumption of Fen treated mice (Pawan 1970), and 5% increases in the metabolic rate in obese and non-obese humans with Fen (particularly after a carbohydrate meal), (Levitsky and Stallone 1988). Fen stimulates dietary-induced thermogenesis (DIT) in brown adipose tissue (BAT), (Lupein and Bray 1985, Underberger *et al.* 1985). Further support for increased DIT with Fen is shown by Fen stimulating BAT GDP binding and increases the firing rate of sympathetic nerve efferents innervating the BAT (Arase *et al.* 1989). D-Fen reduces the 3 hour thermic effect of feeding (but not resting metabolic rate) in obese patients during very low calorie diets (Recasens *et al.* 1995).

1.4.7. Central effects of Fenfluramine.

Although Fen is structurally similar to amphetamine (AMP), (see figure 1.3 and 1.4) a substituted phenethylamine, their neurochemical actions are very different. Amphetamine exerts its anorectic activity primarily by the stimulation of dopaminergic and β-adrenergic release and receptor stimulation (Leibowitz 1986, Parada *et al.* 1988), whilst Fen, and its primary metabolite, Nor-Fen, act specifically on the serotoninergic system. The racemate of Fen lowers 5-HT and 5-HIAA levels at doses of 3mg kg^{-1} i.p. (Duhault *et al.* 1979, Garattini *et al.* 1979). At the same dose the levels of dopamine metabolites, dihyroxyphenyacetic acid (DOPAC) and homovanillic acid are elevated,

whilst at higher doses, (15mg kg^{-1} i.p.), levels of the NA metabolite, 3-methoxy-4-hydroxyphenylethylene glycol sulphate (MOPEG.SO_4) are elevated (Invernizzi *et al.* 1986). The effect on 5-HT and 5-HIAA lasts several days, whilst the effect on the catecholamines lasts only a few hours (Garattini *et al.* 1975).

As early as 1966, it was noted that the D-(+)- form of fenfluramine (D-Fen) possessed almost all of the anorectic activity of the raceamate. Invernizzi *et al.* (1986) demonstrated the differing action of the two isomers of fenfluramine, by comparing the changes in monoamine levels, metabolism and synthesis in rat brains. D-Fen is more potent than L-Fen in decreasing 5-HT and 5-HIAA. Inhibition of 5-HT production, using decarboxylase inhibitors (hydroxybenzylhydrazine - NSD 1015, Ro4-4602 and MK486), (Invernizzi *et al.* 1986, Leibowitz *et al.* 1988), gives an indication of 5-HT synthesis, by the measurement of precursor (5-HTP) build up. A low dose of D-Fen (2mg kg^{-1} orally) reduces 5-HT synthesis in the hypothalamus and lower brain stem, and at a higher doses (5mg kg^{-1}), decreases 5-HT synthesis in all brain regions. A much higher dose of L-Fen (10mg kg^{-1}) is needed to affect 5-HT synthesis. Metergoline, a non-selective 5-HT_{1A} and 5-HT_{1B} receptor antagonist, does not modify the effects of either D-Fen or L-Fen on 5-HT synthesis. L-Fen (5-20mg kg^{-1}) increases MOPEG.SO_4, homovanillic acid and DOPAC levels. No change in homovanillic acid and DOPAC levels are seen with D-Fen. These results (Invernizzi *et al.* 1986) demonstrate that that the D-Fen isomer of Fen racemate affects 5-HT levels, metabolism and synthesis, with few effects on catecholaminergic levels and metabolism, whilst the opposite is true for L-Fen. Consistent with these findings that D-Fen action involves the stimulation of 5-HT release (Garattini *et al.* 1986, Mennini *et al.* 1985) and the inhibition of re-uptake (Garattini *et al.* 1986, Mennini *et al.* 1985), the 5-HIAA levels, following D-Fen treatment, initially increase (increased metabolism of brain 5-HT due to increased release) and then fall (due to depletion of central 5-HT).

D-Fen and D,L-Fen but not L-Fen, increases ACh levels in the striatum (Garattini 1978). The decrease in DA may be causal to the increases in ACh levels, as DA exerts an inhibitory action on ACh, in the striatum. GABA shows no change following Fen treatment. Hypothalamic m-ENK and β-endorphin are increased following 5 days of 15

mg kg^{-1} day^{-1} D-Fen. This elevation is reversed by methergoline (Harsing *et al.* 1982). The anti-dopaminergic action of L-Fen differs from other typical antidopaminergic agents, such as haloperidol, in that L-Fen fails to raise the levels of 3-methoxytyramine, the extraneuronal metabolite of DA (Invernizzi *et al.* 1986). Transstriatal dialysis has shown that L-Fen increases DA release, raising DOPAC and homovanillic acid (Carruba *et al.* 1986a/b). This action differs to that of AMP, but is similar to that of haloperidol. Pre-treatment, of animals, with gamma-butyrolactone, a blocker of the firing activity of nigrostriatal DA neurones blocks the release of DA induced by L-Fen (and haloperidol), but not AMP. As stated previously, L-Fen is less effective than D-Fen in causing anorexia, and its mechanism of action is somewhat different from that of D-Fen; L-Fen anorexia is not antagonised by the typical anti-serotoninergic agent, metergoline, whereas D-Fen anorexia is. The anorexic effect of L-Fen should, in view of its effects on dopaminergic, noradrenergic and serotoninergic systems, be considered to be different to that of its optical isomer, D-Fen, which is selective to the serotoninergic system. The functional significance of the L-Fen interaction with the catecholaminergic system remains to be clarified, and the reduced anorexic effect of D,L-Fen compared to D-Fen may be due, in part, to an antagonism of the 5-HT actions of D-Fen by the catecholeaminergic actions of L-Fen.

D-Fen and D-Nor-Fen (and 5-HT) decrease PVN injection of NA induced feeding (Leibowitz *et al.* 1988). The anorexic effect of D-Fen is unaltered by inhibition of 5-HT synthesis using *p*-chlorophenylalanine (PCPA), (Gibson *et al.* 1993) or by 5-HT re-uptake inhibition using fluoxetine (Raiteri *et al.* 1995). The anorexic effect of D-Nor-Fen is increased following PCPA administration (Gibson *et al.* 1993).

D-Fen antagonises the feeding induced by PVN (or perifornical) injection of NPY (Leibowitz 1991, Brown and Coscina 1995).

1.4.8. Mechanism of central 5-HT depletion by D-Fen.

Stimulation of 5-HT release.

The stimulation of release of 5-HT by D-Fen and its metabolite, D-Nor-Fen, enhances serotoninergic transmission by increasing the availability of 5-HT at the post-synaptic receptor (Fuller *et al.* 1988). The reduction in 5-HT levels following Fen treatment is taken as evidence that release of 5-HT has occurred without the compensatory mechanisms of replenishment of the pre-synaptic storage vesicles (by synthesis and/or re-uptake).

Multiple high-dose regimens (Kleven and Seiden 1988, Zaczek *et al.* 1990) and single doses (Clineschmidt *et al.* 1978, Fuller *et al.* 1978b, Rowland 1986) of fenfluramine cause reductions in brain 5-HT and 5-HIAA, with a reduction in the ratio 5-HT:5-HIAA (Fuller *et al.* 1978, Orosco *et al.* 1984, Rowland 1986). Rowland (1986) demonstrated a reduction in 5-HT and 5-HIAA levels to 90% and 45% of the control animal values respectively with a low dose of D-Fen (3mg kg^{-1} day^{-1} for 6 days via miniosmotic pumps), with an increase in the 5-HT:5-HIAA ratio to 200% control values. A higher dosage of D-Fen (6mg kg^{-1} day^{-1} for 6 days via miniosmotic pumps) results in a reduction in the 5-HT and 5-HIAA levels to 35% and 40% of the control animal values respectively, with an slight decrease in the 5-HT:5-HIAA ratio compared to control values. Microdialysis studies (Chiara 1990, Schwartz *et al.* 1989a/b, Shimizo and Bray 1989) indicate that the D-Fen stimulates the release of 5-HT, measured in the extracellular dialysis fluid (in the lateral hypothalamus, hippocampus, frontal cortex and nucleus accumbens), and is seen to produce a maximal response at 30-60 minutes following injection (Laferrere and Wurtman 1989). Short-term therapy with D-Fen shows that 5-HIAA level depletion lag behind that of 5-HT depletion (Duhault *et al.* 1981). Extracellular 5-HIAA levels, measured by differential pulse voltammetry, are increased 15-70 minutes following D-Fen (but not L-Fen) administration and then fall to lower than pre-treatment levels (De Simoni *et al.* 1985).

Using in vitro synaptosomes, the D-Fen isomer is more potent than the L-Fen isomer in stimulating the release of central 5-HT (Garattini *et al.* 1988). D-Fen has a SC_{25} value

of 5μM, whilst L-Fen demonstrates a SC_{25} value of 3μM (the SC_{25} values for D-Nor-Fen and L-Nor-Fen are 1 and 2μM respectively). The SC_{25} values are the concentration of a drug necessary to cause the stimulation of [^{3}H]5-HT from loaded synaptosomes by 25% of maxima. The mechanism of action of D-Nor-Fen in the stimulation of 5-HT release, found in the *in vitro* studies, is shown to act differently from D-Fen (Borsini *et al.* 1982, Garattini *et al.* 1988). Pre-treatment of the synaptosomes with reserpine, to deplete 5-HT contained within presynaptic storage granules, attenuates the 5-HT release by D-Fen and enhances 5-HT release by D-Nor-Fen. (Borroni *et al.* 1983, Mennini *et al.* 1981). The SC_{25} values for 5-HT release in reserpine-treated synaptosomes is >100μM and 0.2μM for D-Fen and D-Nor-Fen respectively. This suggests that D-Nor-Fen stimulates 5-HT release from a reserpine-insensitive extragranular pool independently to the D-Fen stimulated 5-HT release. Fenfluramine-stimulation of release is calcium-independent (Langer and Moret 1982), which shows that impulse input to presynaptic 5-HT neurones or presynaptic auto-receptors does not mediate this action.

Inhibition of 5-HT uptake.

The action of D-Fen in potentiating 5-HT release is synergistically enhanced by the ability of Fen to cause an inhibition of the 5-HT high-affinity re-uptake into the presynaptic knob (Angel *et al.* 1988, Fuller *et al.* 1988). Re-uptake is the major route of 5-HT inactivation and so inhibition of this uptake, together with the increased release, would maintain the synaptic concentration further following Fen treatment, and so prolonging post-synaptic receptor occupancy. Garattini *et al.* (1988), showed, using 5-HT synaptosomes, that D-Fen and D-Nor-Fen inhibits re-uptake. The IC_{50} values of D-Fen and D-Nor-Fen, in inhibiting 5-HT re-uptake are 0.5μM and 1.4μM respectively (the IC_{50} values of L-Fen and L-Nor-Fen are 8.9μM and 14.0μM respectively). The IC_{50} values of D-Fen and D-Nor-Fen, in inhibiting NA re-uptake are 10.0μM and 1.8μM respectively (the IC_{50} values of L-Fen and L-Nor-Fen are 13.0μM and 17.8μM respectively). D-Fen and L-Fen (and their metabolites) have only a small effect on the uptake of DA into synaptosomes. The mechanism of action for blocking of 5-HT uptake is thought to be different to another 5-HT uptake blocker, fluoxetine, as D-Fen-stimulated 5-HT release can be blocked by fluoxetine but not vice versa (Clineschmidt

et al. 1978, Fuller *et al.* 1978b, Harvey 1978, Steranka and Saunders-Bush 1979). Fluoxetine inhibits the re-uptake of 5-HT by direct antagonism for the uptake receptor whilst D-Fen is transported into the neurone by the same carrier for 5-HT.

These results suggest that D-Fen and D-Nor-Fen are specific in their inhibition of 5-HT re-uptake, and that L-Fen and L-Nor-Fen have very weak inhibitory actions.

Binding to 5-HT receptors.

With the exception of D-Nor-Fen for the 5-HT_2 (specifically 5-HT_{2C}) receptor, the direct effect of D-Fen and D-Nor-Fen on the binding of various ligands on 5-HT subtype receptors are weak (Offermier and du Preez 1978, Garattini *et al.* 1988).

The effect of D-Fen on 5-HT_{1A} receptors is about 7 times less than its inhibitory action on 5-HT uptake. Antagonism of D-Fen using specific a 5-HT_2 receptor antagonist, ritanserin (Leysen *et al.* 1982), and metergoline, a non-specific competitive antagonist with equipotent affinities for 5-HT_1 and 5-HT_2 receptors (Peroutka *et al.* 1981, De Blasi and Mennini 1983) reveals no effect by the former in antagonising the anorexia produced by D-Fen, whilst metergoline antagonises the D-Fen anorexia. This result suggests that the 5-HT_2 receptor is not involved in the mediation of the D-Fen induced anorexia. A method to establish the role of either 5-HT_{1A} or the 5-HT_{1B} receptor in the effect of D-Fen is to discover which receptor is regulated by the administration of the drug. Synaptic regulation following the decrease of a neurotransmitter release classically produces a post-synaptic supersensitivity. A decrease or increase in the density (B_{max}) of a receptor subtype without changes in the affinity (K_a), usually indicates a down- or up-regulation of receptor numbers respectively. D-Fen (5.0mg kg^{-1} day^{-1} for 4 weeks i.p.) reduces the B_{max} of 5-HT_1 and 5-HT_2 receptors to the same extent as a 5-HT direct agonist (selective 5-HT_{2C}), *m*-chlorophenyl-piperazine (*m*CPP) and differently from the 5-HT antagonist metergoline. No changes in 5-HT_{1A} subtypes are found with the ligand [^{3}H]8-OH DPAT (Garattini *et al.* 1988), even though there is a net reduction in brain 5-HT_1 receptor numbers. These results suggest that the 5-HT_{1B} receptors are down regulated following repeated D-Fen therapy. The specificity of D-Fen is demonstrated by the inability of L-Fen to affect 5-HT receptor subtypes

(Garattini *et al.* 1988). 5-HT_{1A}/5-HT_{1B} and 5-HT_{1D} receptor antagonism using WAY100635 and GR127935 does not reduce the anorexic effect of Fen (Hartley 1995).

Section 1.4.7 demonstrated that the central effects of D-Fen and D-Nor-Fen are mediated by different mechanisms. The D-Nor-Fen, but not D-Fen, anorectic effect appears similar to that of the 5-HT_{2C} agonist, *m*CPP, as indicated by the relative potencies of antagonists and the lack of effect in mice lacking the 5-HT_{2C} receptor (Mennini *et al.* 1991, Kennett and Curzon 1991, Tecott *et al.* 1995). PCPA increases the anorexic effect of D-Nor-Fen and *m*CPP (but not D-Fen). Chronic *m*CPP treatment attenuates D-Nor-Fen (but not D-Fen) induced hypophagia, (Kennedy *et al.* 1993). Antagonism of 5-HT_{2C} receptors using 1-napthylpiperazin inhibits the anorexic effect of D-Nor-Fen (and *m*CPP) but not D-Fen (Gibson *et al.* 1993).

Thus we can see a separate serotoninergic effect of D-Fen, a D-Fen stimulation of 5-HT release and a D-Nor-Fen activation of 5-HT_{2C} receptors.

5-HT synthesis.

Fen decreases the rate of 5-HT synthesis (Clineschmidt *et al.* 1978). The activity of the enzyme tryptophan hydroxylase is reduced following Fen treatment (Steranka and Saunders-Bush 1979), although some groups show no changes (Le Douarec and Neveu 1970). The bioavailability of the precursor to 5-HT, tryptophan, is unchanged following Fen administration (Costa *et al.* 1971). The change in 5-HT synthesis is not thought to be limited by the conversion of tryptophan to 5-HT, by tryptophan hydroxylase, as giving a tryptophan load to Fen treated animals causes a restoration of the depleted 5-HT levels, which indicates that the activity of tryptophan hydroxylase is not altered (Rowland and Carlton 1986), and tryptophan hydroxylase is not saturated at physiological concentrations of tryptophan (Fernstrom 1976, Gibson and Wurtman 1978).

D-Fen receptor binding.

A specific reversible D-Fen binding site is found in many brain areas, with particular concentration in regions of serotoninergic (Gobbi *et al.* 1989). Work is underway to characterise this binding and establish a functional correlate to D-Fen action (Campbell 1991).

Neurotoxicity.

The observations that significant 5-HT depletion occurs from a single high dose of Fen can be seen up to 30 days later (Harvey and McMaster 1975), and the reductions from multiple dosages providing depletion lasting for months (Clineschmidt *et al.* 1978, Duhault and Vernavainne 1967, Steranka and Saunders-Bush 1979, Appel *et al.* 1991) led to the possibility of Fen having a neurotoxic effect upon 5-HT neurones. Molliver and Molliver (1990) and Harvey (1978) demonstrated that low doses of D-Fen (5 and 10mg kg^{-1} day^{-1}) given to rats, produced a reduction of fine 5-HT neurones in the neocortex, striatum, hippocampus and medial hypothalamus, whilst sparing 5-HT neurones in the brainstem, LH, raphe nucleus and dentate gyrus (which all possess large spherical varicosities). No decrease in 5-HT markers is seen with L-Fen or L-Nor-Fen administration (Johnson and Nichols 1990). D-Nor-Fen was found to cause a significantly greater decrease in 5-HT markers compared to D-Fen. These anatomical results may explain the depletion of 5-HT and 5-HIAA associated with chronic Fen treatment. Recent studies, however, have challenged the association of D-Fen and a neurotoxic effect.

These results suggest that the mode of the serotoninergic action of D-Fen (and D-Nor-Fen) is mediated by the following mechanisms:

- **Increased 5-HT release**
- **Decreased 5-HT re-uptake**
- **Acting via the 5-HT_{2C} receptor**
- **Direct binding to D-Fen receptor**
- **Neurotoxicity to serotoninergic neurones (5-HT neurone lysis/release possibly mediated via D-Fen metabolite, D-Nor-Fen).**

1.4.9. Effects of serotoninergic lesioning on Fenfluramine anorexia.

The effect of serotoninergic nerve lesioning on Fen anorexia is confused. It would seem probable to assume that if Fen anorexia is a resultant of stimulation through a serotoninergic system, then destruction of 5-HT neurones should attenuate or abolish this effect. Breisch *et al.* (1976) demonstrated that central 5-HT depletion with *p*-chlorophenyalanine (PCPA) causes hyperphagia and obesity, 5,7-DHT also induces hyperphagia in rats (Saller and Strickler 1976). Fen anorexia is abolished with electrocoagulation of median eminence nuclei (Samanin *et al.* 1972). Other studies, however, have failed to reproduce these results using dorsal and medial radiofrequency coagulation or 5,7-DHT brain lesioning (Carey 1976, Hollister *et al.* 1975, Fuxe *et al.* 1975 Carlton and Rowland 1984). 5,7-DHT, in conjunction with desimipramine, produces large reductions in brain 5-HT compared to 5,6-DHT and produces a small enhancement of Fen anorexia, without any change of Fen uptake into brain tissue (Mennini *et al.* 1980). 5,7-DHT and raphe electrocoagulation lesions produces a reduction in plasma corticosterone stimulated by D-Fen (Schetti *et al.* 1979). 5,7-DHT has no effect on CRF release by D-Fen. Other groups show that attenuation of Fen anorexia by neurotoxin (Clineschmidt 1973) and electrolytic lesions (Davies *et al.* 1983, Samanin *et al.* 1972), and its action is not affected by destruction of central catecholaminergic containing systems (Davies *et al.* 1983, Samanin *et al.* 1972).

The confliction in the results may be a result of a difference between deinnervation supersentivity occurring between the studies. So, differences in the extent of the supersensitivity may result in either a reduced, similar or even an increased response when 5-HT is released from the remaining nerve terminals, depending on the extent of post-synaptic supersentivity. The extent of supersensitivity within discrete brain areas is not known, except that in the spinal cord, following 5,7-DHT lesions, there is an increase in motor syndrome to the application of 5-HT and 5-HT agonists (Carlton and Rowland 1984).The depletion of 5-HT following lesions with 5,6-DHT and 5,7-DHT are not uniform (Carlton and Rowland 1984).

Lesions of the dopaminergic system, using nigral injections 6-OH DA in animals pre-treated with tranylcypromine (a MAO inhibitor) have no effect on Fen anorexia (Heffner and Seiden 1979, Hollister *et al.* 1975).

Lesions to the LH enhance Fen anorexia (Blundell and Leshem 1974, Blundell and Leshem 1975), contrary to the abolition or reduction seen with amphetamine anorexia (Fibiger *et al.* 1973, Ahlskog *et al.* 1984). Lesions to the VMH (and the anterior hypothalamus and preoptic area) do not affect the Fen anorexia (Bernier *et al.* 1969, Blundell and Leshem 1975).

1.5. The Autonomic Nervous system.

The previous sections reviewed the effects of various manipulations on the efferent central signals of the autonomic nervous system (ANS). Bray (1989a) classifies the control of energy intake to be a feedback system of two compartmental systems, of a controller (brain) and the peripheral action organs (BAT, muscle, liver, gastrointestinal tract). The previous sections dealt with the feedback regulation of energy intake from afferent peripheral signals, and the central controller actions. Afferent signals to the central controller include hormones such as insulin, glucagon and glucocorticoids, peripheral nutrient and metabolite levels, such as glucose, amino acids, ketone bodies and fatty acids in the plasma and neural efferent signals from the gut and liver. The regulation of the autonomic nervous system (ANS) determines the mode of action in terms of the metabolic fate of ingested nutrients (Bray 1986, Dallman and Bray 1986). The sympathetic nervous system (SNS) arm of the ANS, in conditions that are associated with obesity, including VMH lesions and the listed genetic obesities (*db/db*, *fa/fa*, *ob/ob*), display reduced and blunted activities (Sakaguchi *et al.* 1988), whilst the converse is true for conditions that are associated with body weight loss and hypophagia, including LH lesions, a high carbohydrate diet, adrenalectomy and CRF administration centrally (lesions to the PVN, parasaggital knife cuts, and lesions of the ventromedial NA bundle, which produce obesity by hyperphagia have no effect on the SNS activity), (Bray *et al.* 1989a). The parasympathatic nervous system (PSNS) arm of the ANS is increased and reduced the obesities and conditions associated with weight loss respectively. A negative correlation between the activity of the SNS and feeding exists, shown by the relationship between the basal firing rate of sympathetic nerves to BAT (Bray *et al.* 1989a). Basal sympathetic firing rate is highest, in rats, at 1200-1600 hours, when food intake is lowest, and lowest at 2400 hours, when food intake is highest (Sakaguchi *et al.* 1988).

1.5.1. The Autonomic and Endocrine Hypothesis.

The reciprocal actions of the LH and the VMH regions of the hypothalamus led Bray and York to hypothesise their Autonomic and Endocrine hypothesis (Bray 1986, Bray and York 1979, Bray 1989a). Lesions of the VMH, as stated, produce obesity by a reduction in the SNS drive to BAT, an increase in the PSNS activity to the pancreas and hyperphagia. The converse is true for lesioning to the LH region of the hypothalamus, which results in weight loss, stimulation of the SNS, reduction in the activity of the PSNS and hypophagia (Arase *et al.* 1987). Stimulation of the VMH and LH area electrically produces opposite effects, resulting in body weight loss and increase, respectively. The previous sections dealt with the effects of circulating hormones, metabolites and fuels on the modulation of nutrient balance. The hypothalamic area seems to be the most important area for the integration of these signals to modulate the balance between food intake and energy expenditure. It is from the observations of various manipulations to the hypothalamus, the effect of neurotransmitters on the hypothalamus and alterations of neurotransmitters associated with the feeding response (noticed primarily in the hypothalamus), that the hypothalamic region is thought to be the central controller in the regulation of nutrient balance (Bray 1984, Bray *et al.* 1989a). All of the experimental obesities have a decreased activity of the DIT by the SNS, which is activated by the VMH and inhibited by the LH.

1.6. Obesity.

The study of nutrient selection enhances our understanding of the aetiology of nutritional disturbances, and enables the development of nutritional protocols for the treatment of obesity to be evaluated. Obesity not only presents the obvious mobility incapacity, but also presents risk from life-threatening cardiovascular diseases (hypertension, atherosclerosis) and non insulin-dependent diabetes mellitus as the index of obesity increases (Renold 1981).

Human obesities can be categorised into either;

- Neuroendocrine caused by, for example, hypothalamic injury (Powley and Opsahl 1974, Bray 1984, Sakaguchi and Bray 1988), Cushing's syndrome, ovarian cysts, hypogonadism, GH deficiency, insulinoma and hyperinsulinaemia.
- Nutritional intake imbalance (Bray 1989b).
- Drug-induced by such agents as tricyclic antidepressants, glucocorticoids, phenothiazines, cyproheptadine and lithium compounds (Bernstein 1987).
- Genetic, in such syndromes as Prader-Willi, Bardet-Biedl, Alstrom, Cohen and Carpenter.
- Physical inactivity caused by job-related or enforced situations.

1.6.1. Treatment of obesity.

The treatment of obesity, not associated with endocrine or pathological situations, is to either reduce the energy intake of an individual and/or increase the energy expenditure (see equation on page 1). Methods include counselling (Weight Watchers), very low calorie diets (VLCD's), (Contaldo *et al* 1981, Howard 1981), exercise with caloric restriction (Segal and Pi-Sunyer 1989), surgical techniques (jaw wiring, jejuno-ileal bypass, gastric stapling), (Bray 1989b, Pilkington 1980, Mason 1980, Kral 1989, Kral and Görtz 1981) and pharmacological manipulations. The main drug therapies that exist are bulk forming agents e.g. methylcellulose, thermogenic drugs and centrally acting appetite suppressants. The study of centrally acting drugs enables important

information to be gathered on central interactions in the modulation of body weight and food intake. Centrally active drugs, used to reduce body weight, include D-amphetamine, fenfluramine (racemate)/D-fenfluramine (see section 1.4), fluoxetine, sertaline, phentermine, diethylpropion, mazindol and phenypropanolamine. Fenfluramine has provided an important tool for the investigation of the role of serotonin (5-HT) in nutrient selection in both humans and animal models. The sections 1.1.2, 1.1.3 and 1.1.4. detail the actions of fenfluramine and neurotransmitters, and the balance of nutrient selection.

1.6.2. Use of animal models in the study of obesity and nutrient intake.

Models of obesity in animal models include;

Dietary.

Obesity, in the laboratory (and in humans), can be produced by providing animals with high fat diets (Salmon and Flatt 1985, Schemmel *et al.* 1970), introducing sucrose into drinking water (Kanarek and Hirsch 1977, Kanarek and Orthen-Gambill 1982) or by the presentation of a cafeteria/supermarket diet, which consists of highly palatable carbohydrate and fat rich sweets and snacks (chocolates, sweets, cakes, cheese, peanuts, marshmallows etc.), (Hill *et al.* 1984, Gianotti *et al.* 1988, Rothwell *et al.* 1982, Rothwell and Stock 1986).These manipulations are reversible, and the removal of these diets normally causes a restoration of body weight to the normal chow food intake control animals (Faust *et al.* 1976, 1978, Armitage *et al.* 1983).

Hypothalamic. (see section 1.2.2. on central regions in the modulation of food intake).

Lesions of the ventromedial hypothalamus (VMH), using electrolytic damage (Leibowitz *et al.* 1981, Bray and York 1979, King *et al.* 1989), hypothalamic injection of bipiperidyl mustard (Laughton and Powley 1981, Scallett and Olney 1986), monosodium glutamate (Scallett and Olney 1986), gold-thioglucose or aspartate (Burbach *et al.* 1985) produce obesity in experimental animals which is not dependent on the induced hyperphagia (Arase *et al.* 1987, Bray 1987). However the hyperphagia exacerbates the development of the obesity. Lesions of the paraventricular nuclei of the

hypothalamus (PVN) produce obesity in laboratory animals, which is dependent on hyperphagia (Bray *et al.* 1989). Lesions of the lateral hypothalamus (LH) cause a inhibition of feeding in laboratory animals and conversely, LH stimulation produces an obesity associated with hyperphagia. Electrical stimulation of the VMH produces anorexia (Bray *et al.* 1989a).

An advantage of studying hypothalamic-induced obesity or anorexia is that it enables the contribution of factors, other than nutrient intake imbalance, to be evaluated. As seen in the hypothalamic obesities the role of hyperphagia is variable.

Genetic.

Animals that display spontaneous obesity include the yellow mouse ($A^{vy}/a;A^{iy}/a$), adipose mouse (Ad), diabetic mouse (db/db), fat mouse (Fat), PAS mouse, obese mouse (*ob/ob*), fatty (Zucker) rat (*fa/fa*) and the corpulent rat (SHR/N-cp). All these models are hyperphagic (Bray and York 1979). However, the obesity is not dependent on the hyperphagia (Bray *et al.* 1989), which suggests that the mechanism of the obesity induction is primarily a result of decreased energy expenditure in relation to energy intake. A reduction in the metabolic rate (measured by oxygen consumption) has been shown in new born *fa/fa* rats and *db/db* mice (Bray and York 1979, Planche *et al.* 1983, Bazin *et al.* 1984). Obese *fa/fa* rats and *ob/ob* mice (Holt et al. 1983, Knehans and Romsos 1983, Zaror-Brehrens and Himms-Hagen 1983) show normal brown adipose response to cold acclimation. These animals, however, display an inability to respond to the increase in metabolic rate associated with feeding, dietary-induced thermogenesis (DIT), by brown adipose tissue (BAT), (Holt *et al.* 1983, Rothwell *et al.* 1983, Vander Tuig *et al* 1979, Marchington *et al.* 1983). The BAT of these animals display a low capacity for thermogenesis, as indicated by low blood flow, increased fat deposition and low GDP levels (Holt *et al.* 1983, Hogan and Himms-Hagen 1980, Allars *et al.* 1987) but with intact normal sympathetic afferent innervation. Hyperinsulinaemia is associated in all the obese rodent models (Bray and York 1979) and is essential for the insulin resistance of all rodent obesities (Penicaud *et al.* 1987, Grundleger *et al* 1980).The La/Ncp rats, and *db/db*, *ob/ob* and AY/a mice are all

hyperglycaemic (Bray and York 1979), and the *ob/ob* and *db/db* mice show increased ketone bodies, triacyl-glycerols and cholesterol (Bray 1974).

Plasma corticosterone levels, in the early morning, in *fa/fa* rats are elevated compared to lean littermates (Fletcher 1986). Adrenalectomy reduces the development of obesity in *fa/fa*, *ob/ob*, AY/a and *db/db* rodent models (Yukimura and Bray 1978, Saito and Bray 1984, Freedman *et al.* 1985a/b). The suppression of obesity, by the removal of endogenous corticosterone, is thought to result from reduction in food intake to that seen in lean littermates (Saito and Bray 1984, Dubuc and Wilden 1986), reduction (but not quite normalisation) of plasma insulin levels (Fletcher 1986), and the restoration of a fully functional sympathetic nervous system (Van der Tuig *et al.* 1984, York *et al.* 1985). Replacement glucocorticoid therapy to adrenalectomised lean and obese rodent models demonstrates an increased responsiveness to corticosterone in the obese animals (Freedman *et al.* 1985). The activity of the sympathetic nervous system (SNS) in obese rodents is reduced (Knehans and Romsos 1982, Levin *et al.* 1983, Marchington *et al.* 1983, Young and Lansberg 1983). The SNS drive to *fa/fa* BAT is intact, so the decrease in SNS drive seen in *fa/fa* rats is a result of reduced activity under central control and not a defect in SNS afferent pathways. Adrenalectomy increases the decreased SNS activity found in obese rodents (Van der Tuig *et al.* 1984, York *et al.* 1985). The parasympathetic nervous system (PSNS) activity is elevated in *fa/fa* rats (Rohner-Jeanrenaud *et al.* 1983, Stubbs and York 1991) and in the *ob/ob* mouse (Flatt and Bailey 1981).

The process of nutrient and energy balance can be seen to be a multifactorial and multicompartmental process encompassing physiological, biochemical, pharmacological, genetic, psychological and environmental factors, and it is only by the isolation and study and the inter-relationship of these individual factors (with their individual components) and possible malfunctions of these factors, that an understanding of body weight maintenance and thus the aetiology of obesity be evaluated.

1.7. Project Outlines.

This thesis describes the results of a project designed to investigate the interaction of D-Fenfluramine with adrenal glucocorticoids in the regulation of food intake and energy balance. The potentiation of Fenfluramine (racemate) anorexia by adrenalectomy has been documented by Arase *et al.* (1989c).

It seemed logical to investigate this interaction by studying possible loci and a mechanism(s) for the interaction by investigation of the pharmacological and physiological systems associated with the modulation of D-Fenfluramine and adrenalectomy in the reduction of body weight and food intake, and to evaluate the results in terms of possible value for clinical usage.

The systems below were studied to show the interaction of D-Fenfluramine and adrenalectomy:

- **The hypothalamic and hippocampal serotoninergic system;**
- **The efficiency of energy metabolism and changes in body composition;**
- **Hypothalamic CRF dynamics; and**
- **The metabolism of D-Fenfluramine, and elimination of D-Fenfluramine and the primary metabolite of D-Fenfluramine, D-Nor-Fenfluramine.**

The identity of the adrenal factor that influences the response to D-Fenfluramine was investigated.

Chapter 2.

Materials and Methods.

2.1. Chemicals.

All chemicals listed were obtained from Sigma (Poole) or BDH (Poole), with the exception of the following materials:

1,2,6,7 [^{3}H]-corticosterone ..... Amersham (Buckinghamshire, UK)
D-Fenfluramine* ..... Laboratories Servier (Neuilly, France).
Dextran T70 ..... Pharmacia Fine Chemicals (Uppsala, Sweden)
Glucose oxidase ..... Boehringer Corporation (Lewes, UK)
Norit A® charcoal ..... Aldrich Chemical Co. Ltd (Gillingham, UK)
Optiphase hisafe 3®
aqueous scintillation fluid ..... LKB-Wallac/Fisons (Loughborough, UK)
Peroxidase II ..... Boehringer Corporation (Lewes, UK).
Sodium pentobarbitone
(Sagatal®) ..... May and Baker (Dagenham, Essex, UK)

*D-Fenfluramine specifications (Servier certificate of analysis):

L (-) contamination 2.7%
H_2O 0.15%
Sulphated ash 0.01%
Heavy metals ..... ≤20ppm
Optical specifications ..... $+20° \leq [a]^{22} \leq +23.2$
(Typical 21.1°).
Chloride specification ..... $12.98\% \leq t \leq 13.50\%$
(Typical 13.26%).

Antisera

Goat anti-guinea pig	ICN, Lisle, IN, USA.
Guinea-pig anti bovine insulin	Southampton General Hospital.
Insulin peroxidase conjugate	Sigma (Poole, UK).
Anti-corticosterone-21-thyroglobulin.....	Sigma (Poole, UK).
Goat anti-rabbit.....	Sera Lab. Ltd (Crawley, Sussex).
Rabbit anti-CRF_{41}	University of Reading (Dr. E. Linton).
Rabbit anti-CRF_{36-41}	University of Reading (Dr. E. Linton).
Rabbit anti-CRF_{1-20}	University of Reading (Dr. E. Linton).

Antisera generation

Guinea pig anti-bovine insulin serum (Southampton General Hospital) was raised by a priming dose of 50µg bovine insulin in Freund's complete adjuvant injected into Duncan-Hartley strain 2 guinea pigs, with subsequent boosters, every 2 months, for 6 months of 50µg bovine insulin in Freund's incomplete adjuvant. Antibody titre was assessed initially by ear puncture, with blood collection by cardiac puncture.

Rabbit anti-CRF_{41} and C-terminal rabbit anti-CRF_{36-41} (University of Reading). Synthetic rat/human-CRF_{41} peptide (1mg) or rat/human-CRF_{36-41} peptide (1mg), was coupled to thyroglobulin (2mg) with glutaraldehyde (10µl of an aqueous 25% (*v/v*) solution) dissolved in 0.1M sodium bicarbonate (0.5ml). The mixture was incubated for 1 hour at 20°C and unreacted groups quenched using 100µl 0.5M glycine. The mixture was desalted on a sephadex G50 column (0.6 x 10cm) using 0.01M sodium bicarbonate containing 0.9% NaCl as column buffer, and made up to 12ml using the column buffer. Antibodies were raised in a New Zealand white rabbit (code rCRF4) immunised with conjugated rat/human-CRF_{41} (25µg) emulsified in Freund's complete adjuvant (1:2 v/v) for the primary injection and emulsified in Freund's incomplete adjuvant for two subsequent booster injections (1:2 *v/v*). 500µl aliquots of neat serum were stored at -20°C.

N-terminal CRF peptide, CRF_{1-20}-NH_2, (0.5mg) was conjugated with 1mg bovine thyroglobulin using 1mg *N*-ethyl-*N'*-(3-diamethylaminopropyl) carbodimide HCl and following the same procedure as for the C-terminal antibodies onwards. The anti-CRF_{1-20} antibody was raised in a guinea pig by immunisation at monthly intervals with conjugated CRF_{1-20} (10μg) emulsified in Freund's complete adjuvant (1:2 *v/v*) for the primary injection and CRF_{1-20} (10μg) emulsified in Freund's incomplete adjuvant for subsequent booster injections (1:2 *v/v*).

The rabbit anti-CRF_{1-41} antiserum (code rCRF4) was used for the immuncytochemistry, whilst the IRMA uses both the N-terminal anti-CRF_{1-20} and the C-terminal anti-CRF_{36-41} antiserum.

2.2. Animals.

All animals were female (except where indicated) albino Wistar rats (6 weeks old at initiation, weighing 120-150g) bred and housed at the Southampton University Medical School. The animals were housed individually in wire bottom cages in rooms maintained at 22 ± 1°C, with a 12-hour light phase 0700-1900. All animals were fed *ad libitum* on pelleted PRD rat chow (Labsure), with the following dietary composition (published manufacturers details):

- Crude oil 2.7%
- Crude Protein.....19.7%
- Crude Fibre.....5.3%
- Calcium.....0.6%
- Phosphorous 0.7%
- Sodium chloride 1.0%
- Metabolisable energy .....2.871kcal g^{-1} / 12.02kJ g^{-1}
- Carbohydrate 53.5%
- Minerals and vitamins.....18.0%

The previous 24 hour food intake (corrected for spillage) and body weights were measured between 0900-1030 hours daily.

2.3. Surgical procedures.

Bilateral adrenalectomies were performed under 50mg kg^{-1} sodium pentobarbitone (Sagatal®) anaesthesia. The back of the animals were shaved and lightly swabbed with chlorhexidine gluconate (ICI, Cheshire, UK) 1.25% (w/v) in 50% ethanol (v/v). Adrenals, together with surrounding fat mass, were removed by a bilateral dorsal flank incisions approximately 5mm below the level of the lowest rib, being careful to avoid damage to the adrenal capsule. Sham adrenalectomy surgery was performed by a similar approach, with the adrenal mass and kidney gently agitated to simulate visceral entry. Body wall wounds were sutured with sterile silk sutures and the skin with wound clips. The success of adrenalectomy was verified at termination by examination of the adrenal bed and the assay of serum corticosterone values below the limits detection of the corticosterone RIA (<1.5μg dl^{-1}).

2.4. Drug Administration.

D-Fen was dissolved in 0.9% sterile saline in sterile containers, to give a final concentration of 10mg ml^{-1} of D-Fen. Rats assigned to the D-Fen treatment groups received 10mg kg^{-1} day^{-1} of D-Fen injected i.p. Saline control animals received 1ml kg^{-1} of sterile isotonic saline injected i.p. Fenfluramine or saline injections were administrated between 1700-1800 hours daily.

Rats designated to received corticosterone replacement (5mg kg^{-1} day^{-1}) animals received corticosterone (s.c.) in an arachis oil suspension (10mg ml^{-1}). All other animals, where appropriate, received arachis oil (s.c.) alone (0.5ml kg^{-1} day^{-1}). Fenfluramine or saline injections and corticosterone or arachis oil injections were administrated between 1700-1800 hours daily, except for the day of surgery, when corticosterone replacement or arachis oil administration immediately followed adrenalectomy or sham-adrenalectomy surgery.

2.5. Termination.

At the termination of each experiment, the rats were killed by decapitation between 1100 and 1400 hours. The final drug administration (D-Fen/saline/corticosterone/ arachis oil, where applicable) that the animals received would have been the previous p.m. administration (approximately 18 hours previous). Immediately after decapitation the brain was removed and the hypothalamus and hippocampus dissected away for HPLC analysis of indoleamines (section 2.7.3.) and trunk blood collected, allowed to clot on ice, serum isolated (3000g for 15 minutes) and stored at -20°C until analysis for glucose (section 2.7.1.), insulin (section 2.7.4.) and corticosterone (section 2.7.5.). The liver and ovarian fat pad were weighed and replaced in the carcass for total carcass analysis. The remaining brain regions were stored at -20°C for analysis of D-Fen and D-Nor-Fen (section 2.7.9.). The remaining carcass weight, minus gastrointestinal tract, was weighed and used for body composition analysis (section 2.7.6).

2.6. Statistical analysis.

All data was analysed by a 2-way ANOVA on SPSS/PC+ statistical data analysis software package. A probability level of $p<0.05$ was taken as significance. Significant values were further analysed by the Newman-Keul's procedure. The results of the ANOVA are displayed (with F values and degrees of freedom) in appendices 1 and 2, for figures and tables respectively. NPAR analysis using Kolmogorov-Smirnov Goodness of fit showed normal data distribution.

2.7. Assay protocols.

2.7.1. Measurement of Serum glucose by glucose oxidase reaction.

Glucose was measured directly by the measurement of the formation of glucose oxide. The glucose was oxidised by glucose oxidase in a reaction that also oxidises water into hydrogen peroxidase. The hydrogen peroxide was reduced back to water by the peroxidase with the oxidation of the added chromophore (ABTS).

Working Reagent	0.8mg Peroxidase
	5mg Glucose oxidase
	100mg ABTS
	in 100ml 0.1M PBS buffer pH 7.0 at room temperature.

Serum (diluted 1:20) and standard solutions of glucose (0 to 75μg ml^{-1} in 15μg increments) were made up 0.1M PBS buffer pH 7.0. 10μl of diluted serum or standard solution was added to microplate wells, and 250μl working reagent added. The plates were incubated for 15 minutes at 37°C, and the absorbance at 620nm read against a working reagent and 0.1M Na_2PO_4 blank on a plate reader (MR 580, Dynatech, Virginia, USA).

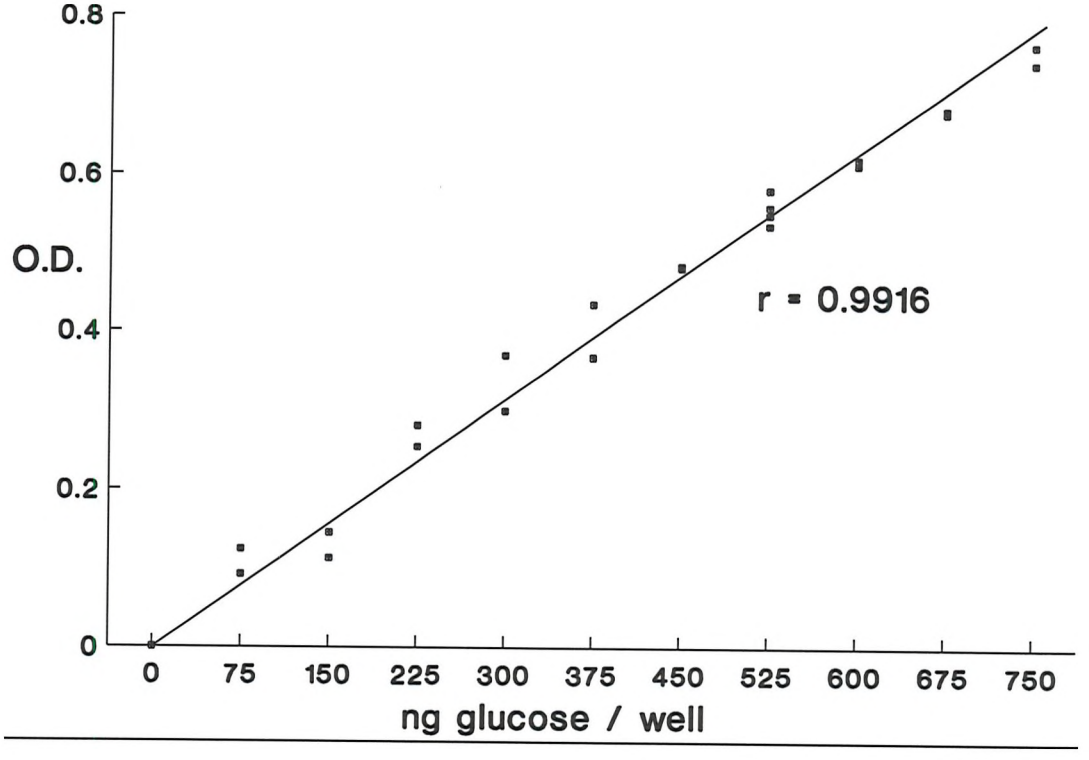
Typical serum glucose levels were approximately 0.9-1.3mg/ml (5-7mM). A typical standard curve is shown in figure 2.1. The coefficients of variation for intra- and inter-assays were 4.3 ± 1.5% and 13.0 ± 7.6% respectively.

2.7.2. Lowry determination of protein.

Lowry Reagent	10ml 2% Na_2CO_3 (*w/v*) in 0.1M NaOH
	0.1ml 1% (*w/v*) Cu_2SO_4
	0.1ml 2% (*w/v*) sodium/potassium tartrate
	1ml 10% (*w/v*) sodium deoxycholate

10µl sample or BSA standard (0-100µg) was added to tubes, and incubated with 1ml Lowry reagent at room temperature for 10 minutes. 0.1ml Folins and Cioceteau reagent added, mixed and incubated at 37°C for 10 minutes. Absorbance was then read at 750nm against a buffer blank on a spectrophotometer (Pye-Unicam SP8-400). The coefficient of variation was 3.14.

Figure 2.1. Glucose standard curve



2.7.3. High-Performance Liquid Chromatography (HPLC) detection of brain 5-hydroxyindoles and Tryptophan.

Serial UV and amperometric detection using a HPLC system established and developed by the author enabled the simultaneous detection of the 5-hydroxyindoles and tryptophan.

Tissue Preparation

Immediately following decapitation the brain was removed from the skull and placed dorsal surface upwards, avoiding contact of neuronal tissue with blood, and the cortex from one side of the brain removed. The hippocampus was removed, by a dorsal approach, from the decorticated region. The brain was then placed ventral side upwards and the hypothalamus dissected. A transverse section was made at the level of the optic chiasm, which delimits the anterior part of the hypothalamus and passes through the anterior commissure. The hypothalamus was dissected by taking the anterior commissure as a horizontal reference (approximately 3mm depth) and the line between the posterior hypothalamus and the mammillary bodies as the caudal limit, and laterally by the hypothalamic fissures. The brain regions were weighed, to the nearest 0.1mg, and quickly homogenised in 200µl 0.3M perchloric acid containing $1x10^{-5}$M HVA, (internal standard) and 0.4mM sodium metabisulphite (antioxidant). The homogenate was then spun at 9000g (12500rpm in 1ml eppendorphs) at 4°C for 20 minutes. The supernatant was aspirated immediately, stored in airtight amber vials and frozen at -70°C until immediately before use.

HPLC system.

The liquid chromatographic system consists of a constametric 3000 solvent delivery system (LDC Analytical, Stone, Staffordshire) with a liquid pulse dampener in series, connected to a 7125 manual Rheodyne (LDC Analytical) with a 20µl injection loop, a C_{18} 37-53µm glass beads Pellicular ODS guard column (Upchurch Sci. C-130B), connected to an apex octadecyl C_{18} reverse phase stainless steel column (25x6.426mm o.d., 4.6mm i.d.), packed with 5µm octadecyl bonded silica spheres (Jones Chromatography, UK). The reverse phase analytes are partitioned between a hydrophobic stationary phase and an aqueous mobile phase. Elution is in the order of increasing hydrophobicity.

The mobile phase was a 0.1M citric-monohydrogen-phosphate buffer, containing 17.5% methanol and 0.4mM sodium metabisulphite at pH 4.5. The mobile phase was filtered and degassed under vacuum, through 0.45µ nylon-66 filters (Rainin Instrument Co.), with helium bubbled continuously through the mobile phase reservoir at

approximately 500ml min^{-1} (to prevent bubble formation in the system). The elution flow rate was adjusted to 1ml min^{-1} (pressure approximately 1700 to 2000 PSI). The HPLC system was run at ambient temperature (typically 18-24°C).

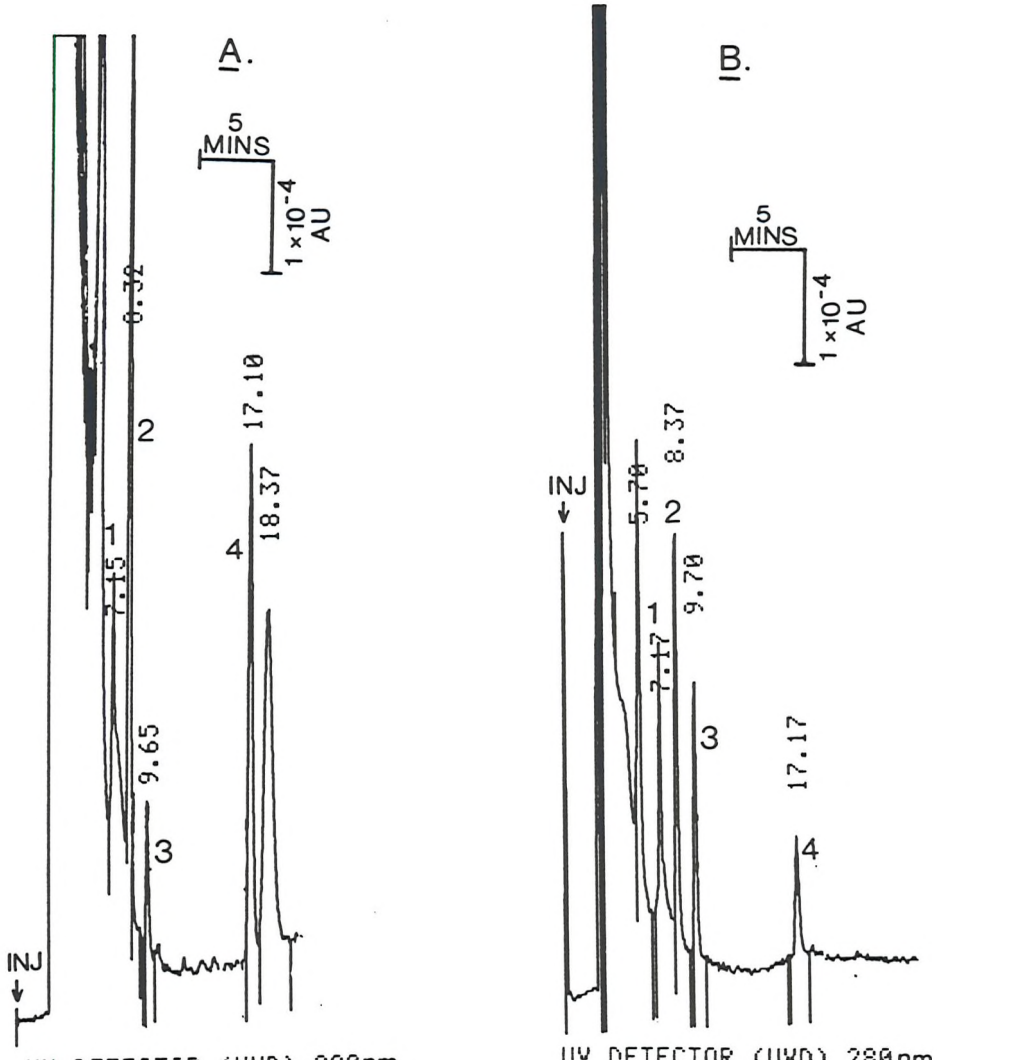
The UV detector (SM 4000 Programmable Wavelength Detector, LDC Analytical) was connected in series, and the wavelength set at 220nm. Connected in series to the UV detector was an electrochemical detector (ECD), (Model 400 Princeton Applied Research). The electrochemical detector consists of a single glassy carbon thin layer flow cell electrode. The reference electrode consists of a silver-silver chloride electrode filled with 3M NaCl/saturated Ag/AgCl, to provide a stable potential with which the working electrode can be compared. The glassy carbon electrode was set at a potential of +600mV verses the reference electrode (Range 10-100nA/V, time constant 5.0 seconds). Air oxidation of indoleamines is a well-known phenomenon, and it is this oxidation that has been harnessed by the electrochemical detector, by carrying out this process on the surface of the working electrode. The oxidation was driven by applying a positive potential, so that the ring hydroxyl groups of 5-HT and 5-HIAA are oxidised to form an orthoquinone derivative with the release of 2 electrons and 2 hydrogen ions. The electrons are transferred to the working electrode, and the resultant current produced is directly proportional to the number of molecules oxidised. The indoles oxidise relatively easily (at +0.5V), whilst the oxidation of tryptophan does not occur until above 0.75mV, when background noise levels are consequently increased. The UV and EC detector signals were integrated using a computer integrator (CI3000, LDC analytical), and stored on disc and displayed on a single channel chart recorder (LDC analytical), following integration. Typical retention times for 5-HT, Tryptophan, 5-HIAA and Homovanillyl alcohol were 7.3, 8.3, 9.8 and 17.2 minutes respectively. Examples of typical UV and EC traces for standard solutions and brain tissue samples are shown in figures 2.2. and 2.3. respectively.

The 5-hydroxyindole and tryptophan losses during the extraction and storage were corrected by calculation of the loss in the concentration of homovanillyl alcohol (HVA), the added internal standard. The tissue weights and dilution were taken into account to estimate the actual tissue 5-hydroxyindole and tryptophan levels.

Figure 2.2.

Typical UV chromatographs (220nm) of:

- **A. 20µl injection mixture of 61mg hypothalamic extract homogenised in 200µl 0.3M perchloric acid, 0.4mM sodium metabisulphite and $1x10^{-5}$M HVA (internal standard).**
- **B. 20µl injection standard solution containing $0.745x10^{-6}$M 5-HT (3.18ng), $0.816x10^{-6}$M 5-HIAA (3.12ng), $1.109x10^{-6}$M HVA (3.73ng) and $1.183x10^{-6}$M tryptophan (4.83ng).**



UV DETECTOR (UVD) 280nm
7.15 MINS (PEAK1) 5-HT
8.32 MINS (PEAK2) TRYPTOPHAN
9.65 MINS (PEAK3) 5-HIAA
17.10 MINS (PEAK4) HVA
(INTERNAL STANDARD)

TIME	AREA
7.15	226554
8.32	468392
9.65	54378
17.10	323837
18.37	446115

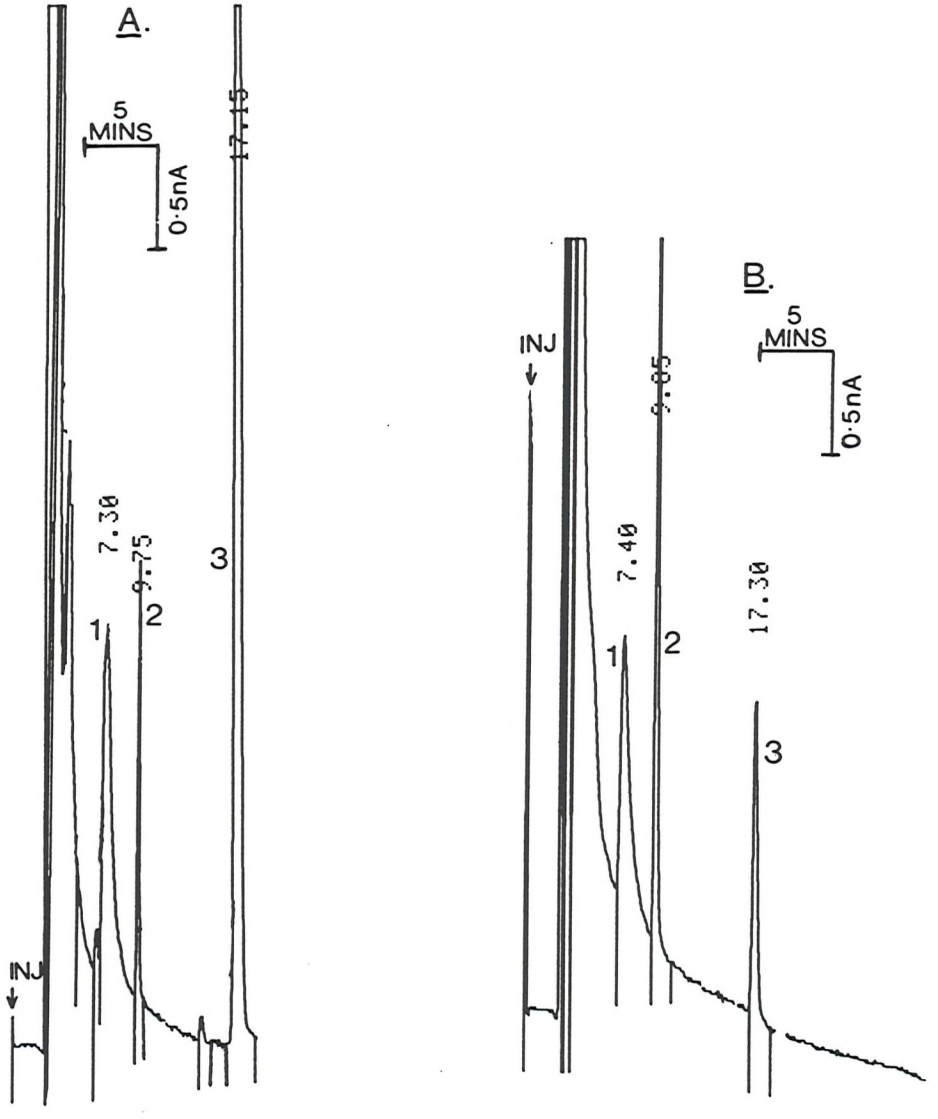
UV DETECTOR (UVD) 280nm
7.17 MINS (PEAK1) 5-HT
8.37 MINS (PEAK2) TRYPTOPHAN
9.70 MINS (PEAK3) 5-HIAA
17.17 MINS (PEAK4) HVA
(INTERNAL STANDARD)

TIME	AREA
7.17	151870
8.37	170746
9.70	111102
17.17	107644
	541362

Figure 2.3.

Typical electrochemical chromatographs (600mV) of:

- **A. 20µl injection mixture of 61mg hypothalamic extract homogenised in 200µl 0.3M perchloric acid, 0.4mM sodium metabisulphite and $1x10^{-5}$M HVA (internal standard).**
- **B. 20µl injection standard solution containing $0.745x10^{-6}$M 5-HT (3.18ng), $0.816x10^{-6}$M 5-HIAA (3.12ng), $1.109x10^{-6}$M HVA (3.73ng) and $1.183x10^{-6}$M tryptophan (4.83ng).**



ELECTROCHEMICAL DETECTOR (ECD)
7.30 MINS (PEAK1) 5-HT
9.75 MINS (PEAK2) 5-HIAA
17.15 MINS (PEAK3) HVA
(INTERNAL STANDARD)

TIME	AREA
7.30	8120006
9.75	3003039
17.15	17170198

ELECTROCHEMICAL DETECTOR (ECD)
7.40 MINS (PEAK1) 5-HT
9.85 MINS (PEAK2) 5-HIAA
17.30 MINS (PEAK3) HVA
(INTERNAL STANDARD)

TIME	AREA
7.40	6130141
9.85	6157400
17.30	3556180

The detection limits of 5-HT, 5-HIAA, HVA and tryptophan, for this system, were 2, 5, 50 and 11pg respectively (quoted as the limit of detection when signal:noise ratio is 3:1). Typical hypothalamic concentrations of 5-hydroxyindoles and tryptophan, using the previous method, would be expected to, from a 20ml injection volume, have approximate levels of 8, 4 and 25ng of 5-HT, 5-HIAA and tryptophan respectively, (Chaouloff *et al.* 1985, Loullis *et al.* 1985, Chance *et al.* 1987).

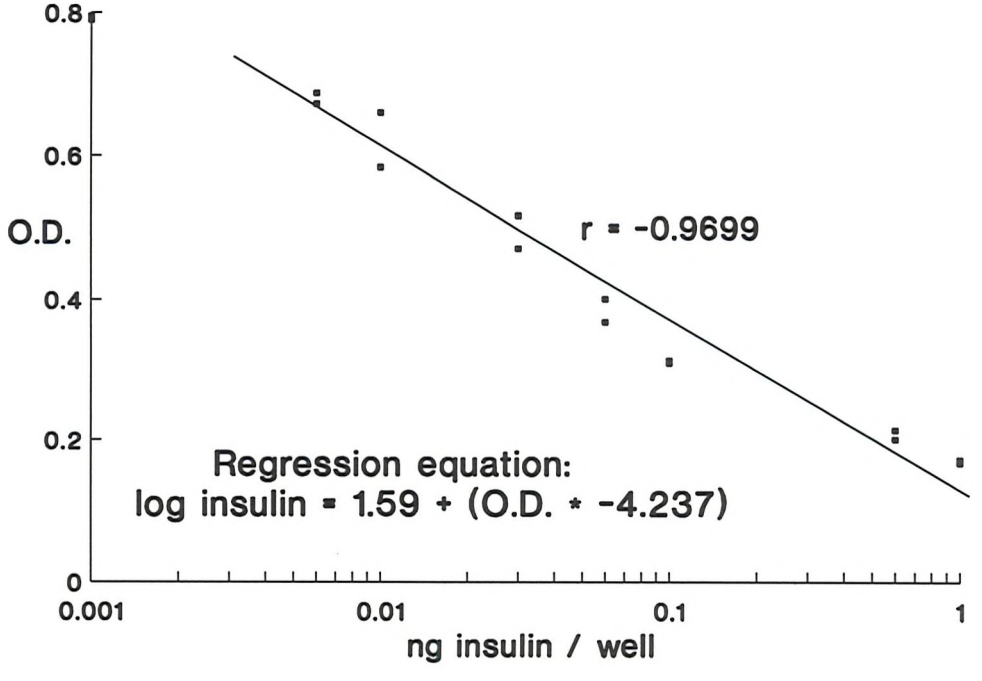
2.7.4. Determination of Serum insulin.

Serum insulin levels were determined using an ELISA method adapted from Kekow *et al.* (1988). The ELISA wells were coated with goat anti-guinea pig antibody, which are then bound to guinea-pig anti-bovine insulin antibodies, which compete competitively for the insulin (from standards or samples) and the insulin-marker enzyme.

Coating buffer (pH 9.6 at 4°C)	59g l^{-1} Na_2CO_3, 2.93g l^{-1} $NaHCO_3$, 200mg l^{-1} sodium azide
Washing buffer (pH 7.2 at 4°C)	8g l^{-1} NaCl, 0.2g l^{-1} KCl, 2.9g l^{-1} $Na_2HPO_4.12H_2O$, 0.2g l^{-1} KH_2PO_4, 0.05% (*v/v*) Tween 20
Coupling buffer (pH 7.4 at 4°C)	40mM phosphate buffer (11.6g l^{-1} $Na_2HPO_4.12H_2O$, 1.18g l^{-1} $NaH_2PO_4.2H_2O$), 1g l^{-1} BSA (RIA grade), 240mg l^{-1} sodium ethylmercurithiosalicylate
Incubation buffer (pH 7.4 at 4°C)	40mM phosphate buffer (11.6g l^{-1} $Na_2HPO_4.12H_2O$, 1.18g l^{-1} $NaH_2PO_4.2H_2O$), 60g l^{-1} BSA (RIA grade) 240mg l^{-1} sodium ethylmercurithiosalicylate, 6g l^{-1} NaCl
o-Phenylenediamine reagent (pH 5.6 at 4°C)	17.9g l^{-1} $Na_2HPO_4.12H_2O$, 7.8g l^{-1} $NaH_2PO_4.2H_2O$, 0.34g l^{-1} *o*-Phenylenediamine, 0.04% (*v/v*) H_2O_2

Microelisa plates (12x8, 250µl wells) were coated with 150µl well^{-1} with goat anti-guinea pig antibody (1mg ml^{-1}) in coating buffer, and incubated for 2 hours at 37°C, and then each well was washed with 1ml of washing buffer. 100µl of guinea-pig anti-bovine insulin antibody (1ml aliquot diluted in 14ml in coupling buffer), was then added to each well, to conjugate with the goat anti-guinea pig antibodies, and incubated for 2 hours at 37°C. The wells were then washed with 1ml washing buffer and then either 100µl of the standards (0, 0.06, 0.1, 0.3, 0.6, 1.0, 3.0 or 10ng ml^{-1} rat insulin in incubation buffer) or serum samples (diluted 1:5 with incubation buffer) were added to each well. The plates were incubated at 4°C for 16 hours, and then 100µl insulin-peroxidase conjugate (1µg ml^{-1} in incubation buffer) was added to each well, and incubated at 4°C for 4 hours. The wells were washed with 1ml of washing buffer, and 100µl *o*-phenylenediamine reagent was added and incubated at room temperature for 30 minutes. The reaction was terminated by the addition of 100µl 0.5M H_2SO_4, and the ELISA plate read on a plate reader (MR 580, Dynatech, Virginia, USA) set at 492nm. The linear regression line from a plot of standard absorbance readings against log insulin concentration then gave the equation for estimation of serum insulin levels.

Figure 2.4. Insulin RIA standard curve



The coefficients of variation for intra- and inter- assays were 6.2 ± 1.1% and 14.0%. Insulin levels are quoted as μU ml^{-1} (1ng ml^{-1} approximates to 23.4μU ml^{-1} or 172pM rat insulin). A typical standard curve is shown in figure 2.4.

2.7.5. Serum corticosterone Radioimmunoassay (RIA).

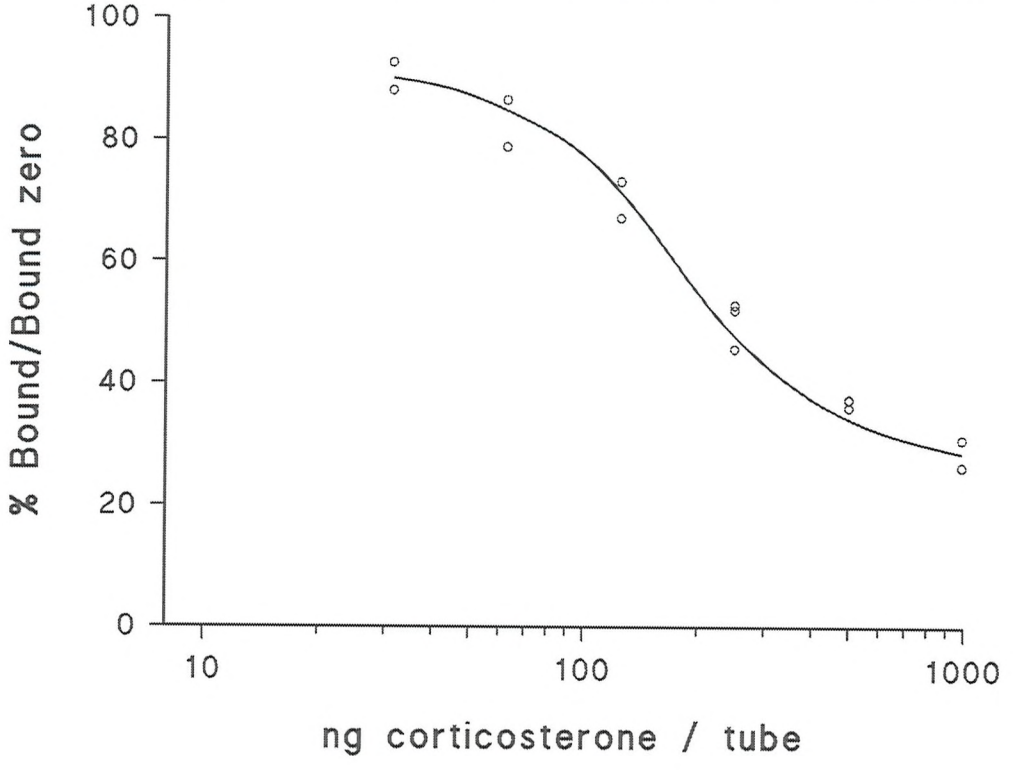
Serum corticosterone levels were determined using competition between ^{3}H-corticosterone and unlabelled corticosterone, in standards or samples, for binding to an anti-corticosterone antibody. Corticosterone not bound to antibody was absorbed by dextran-activated charcoal. Increasing concentrations of corticosterone in the samples or standards competitively displaces bound ^{3}H-corticosterone from the anti-corticosterone antibody in the supernatant, leading to increasing ^{3}H-counts in the charcoal pellets (containing unbound corticosterone i.e. ^{3}H-corticosterone and unlabelled corticosterone)

RIA Buffer	0.05M Tris-HCl, 0.1M NaCl, 15mM NaN_3
RIA Diluent	0.05M Tris-HCl, 0.1M NaCl, 15mM NaN_3, 0.1% BSA
Antiserum	anti-corticosterone-21-thyroglobulin (lyophilised) reconstituted in 5ml RIA buffer, then 45ml RIA diluent.
^{3}H-corticosterone	3.7μml 1,2,6,7-[^{3}H]-corticosterone (75Cimmol^{-1}, 1mCi ml^{-1}) dried under N_2 and 100ml RIA diluent added.
Dextran-coated charcoal	0.05% (*w/v*) dextran T-70, 0.5% (*w/v*) Norit A charcoal stirred for at least 1 hour on ice before use.

50μl of serum samples were extracted, to remove plasma-binding proteins, by the addition of 250μl absolute alcohol, mixed for 5 minutes and centrifuged for 10 minutes. Two 100μl aliquots were removed and dried under N_2. Corticosterone standards (0, 0.625, 1.25, 2.5, 5 and 10ng ml^{-1}) were prepared from 1μg ml^{-1} corticosterone dissolved in ethanol, and diluted accordingly with RIA diluent. 100μl of diluent (200μl to total tubes), 500μl of anti-corticosterone was added (to all except

blanks, where 100µl diluent was added). Tubes were then incubated at room temperature for 30 minutes. 100µl of reconstituted ^{3}H-corticosterone was added and the tubes incubated at 37°C for 1 hour. The tubes were then cooled, on ice, and 200µl of the dextran-activated charcoal suspension added to all tubes (except total tubes, where 200µl diluent was added). The tubes were mixed vigorously and left on ice for 10 minutes. The tubes were centrifuged (1500g for 15 minutes at 4°C). 250µl of the supernatant was removed and 5ml Optiphase aqueous scintillation fluid added. ^{3}H-counts were measured on a liquid scintillation counter (Raytest 4700) for 5 minutes. A typical standard curve is shown in figure 2.5.

Figure 2.5. Corticosterone RIA standard curve.



2.7.6. Body composition analysis.

At termination body carcasses (minus brain, approximately 5ml blood and gastrointestinal tract) were weighed, combined with 2 parts anhydrous sodium sulphate (w/w) and dried for 100 hours at 75°C, in a vacuum oven. The dried animal and sodium

sulphate mixture was then minced using a manual butchers mincer to produce a fine homogenous mixture. This mix was then analysed for carcass body fat, protein and energy content.

Protein content.

Total carcass protein was analysed using a Kjeldahl 1030 auto analyser. Approximately 1g (weighed to the nearest mg) of mixture, in nitrogen-free weighing boats (Schleicher and Schnell/Anderman and Co., Kingston-on-Thames, Surrey, UK), was added to boiling tubes, together with 3ml conc. H_2SO_4, 1 $CuSO_4$ Kjeltab CQ catalyst tablet (Thompson and Copper Ltd, Runcorn, Cheshire, UK) and 0.5ml antifoam A silicon concentrate (BDH). The mixture was then boiled at 420°C for 40 minutes (until a clear green solution is formed), and allowed to cool to room temperature.

The added H_2SO_4 converts nitrogen in the sample (from protein sources) to ammonium sulphate. The ammonium sulphate is then converted to ammonia, by the actions of 40% NaOH, and the liberated ammonia titrated against 0.05M H_2SO_4. Steam distillation, using 2% Na_2SO_4 (*w/v*), distils the ammonia over into the indicator solution. The indicator solution contained 0.1% bromocresol green (w/v) and 0.07% (w/v) methyl red in methanol.

The samples were compared to a standard solution of ammonium sulphate, 33.01g l^{-1}, which corresponds to 7.0mg N ml^{-1}. The % nitrogen was assessed using the formula:

%N = Mw Nitrogen x Normality of the acid x mls acid

1ml of 7mg nitrogen ml^{-1} would yield a titration value of 5ml 0.05M H_2SO_4. The Kjeldahl auto analyser was not used until a reasonable degree of accuracy and reproducibility was obtained.

Calculation of the % nitrogen in the animal mixture was obtained using the equation:

$$\%N = \frac{\text{Mw Nitrogen x Normality of the acid x mls acid}}{\text{weight sample (g)}}$$

Conversion to protein (by multiplication by 6.25), gives mg protein g mixture^{-1}. This was then converted to g protein g wet body weight^{-1}, by taking into account the dilution factor by the sodium sulphate and the water loss during drying. The coefficient of variation was 2.71.

Lipid content.

Approximately 3g dried sample, weighed to the nearest mg, was added to 30mm x 80mm dried cellulose extraction thimbles, weighed to the nearest mg, (Whatman), and plugged with dried non-absorbent cotton wool, weighed to the nearest mg. The thimble was refluxed at 60-80°C with petroleum ether for 6 hours. The final dried weight of the thimble gives an indication of the total ether extractable lipid g dry sample^{-1}. This was then converted to g lipid body weight^{-1}, by taking into account the dilution factor by the sodium sulphate and the water loss during drying. The coefficient of variation was 4.01.

Total combustible energy content.

The combustible energy content of the dried mixture was assessed using a ballistic bomb calorimeter developed by Fox, Miller and Payne (1959) and improved by Miller and Payne (1959). The energy content was quantified using a known weight of the universal standard, benzoic acid. The samples and standards were combusted at 25 atmospheres oxygen, and the temperature change was recorded by a thermocouple, which was then displayed on a galvanometer. Benzoic acid, upon combustion, yields 26434 ± 4J g^{-1}. The calibration constant (KJ/ unit deflection) was calculated as:

$$\textbf{Calibration constant (KJ unit deflection}^{-1}\textbf{)} = \frac{\textbf{26.434 x mass benzoic acid (g)}}{\textbf{Deflection - Blank}}$$

The blank deflection value corresponded to the deflection obtained using the 7cm of ignition string only.

The energy content of the mixture was obtained using the equation:

$$\textbf{Energy content of sample (KJ g}^{-1}\textbf{)} = \frac{\textbf{(Deflection-Blank) x Calibration Constant}}{\textbf{Weight sample (g)}}$$

The value obtained gives the energy content g dry sample mixture^{-1}. This was then converted to energy g wet body weight^{-1}, by taking into account the dilution factor by the sodium sulphate and the water loss during drying. The coefficient of variation was 3.59.

2.7.7. Immunocytochemical detection of CRF_{1-41} in the rat brain.

In this unlabelled antibody enzyme bridge method, described first by Mason *et al.* (1969), primary rabbit anti-(rat/human)-CRF_{1-41} antibody (RACRF) binds directly to the recognition portion of tissue CRF_{1-41} peptide. Rabbit anti-peroxidase antibody is bound indirectly to the primary antibody using, as a bridge, unlabelled goat anti-rabbit IgG (GAR) in excess. The variable region (Fab regions) of the anti-peroxidase rabbit antibody (RAP) bind free peroxidase and the whole complex is developed with DAB/H_2O_2 as substrates. The system is optimised by combining the last two stages of the unlabelled antibody enzyme bridge method (RAP and free peroxidase) by reacting together to form a soluble peroxidase-anti-peroxidase "*PAP*" complex. This "*PAP*" complex acts as the final stage in the antigenic detection that is linked to the RACRF with GAR. The peroxidase substrate, 3,3'-diaminobenzidine tetrahydrochloride (DAB), develops the bound complex by the formation of a brown precipitate. This antibody complex is shown in figure 2.6.

Tissue preparation.

Rats were perfused with 250ml ice cold phosphate buffered saline (PBS) by transcardial perfusion via cannula placement into the left ventricle of the heart, followed by 250ml ice cold 4% formaldehyde in PBS containing 15% (*v/v*) saturated picric acid buffered to pH 6.0 with 0.1M sodium acetate (8.2g l^{-1}), and finally 250ml of the same fixative components buffered to pH 9.5 with 0.1M sodium borate (6.2g l^{-1}). The brain was then removed from the skull and immersed in the alkaline fixative for 24 hours at 4°C. Picric acid was used in conjunction with the formaldehyde in the fixative, as it is thought to improve preservation of the peptide antigens (Somogyi and Takagi 1982) by precipitation and combining to form picrates and intermolecular salt links.

Brains were then transferred to 20% sucrose (*w/v*) in PBS solution for 24 hours at 4°C to provide cryostatic protection.

A 4% formaldehyde solution was prepared by heat depolymerisation of paraformaldehyde. 40g of paraformaldehyde was added to 500ml water and heated to 75-80°C and 2-3ml of 5M NaOH was added slowly until the solution clears, and filtered if necessary. This 8% formaldehyde solution was used to make the final 4% formaldehyde and 15% picric acid solution.

The brain was mounted onto a cryostat chuck using O.C.T. tissue-tek tissue embedding medium (Miles, Elkhart, Illinois, USA) and frozen using liquid nitrogen cooled *iso*-pentane (methyl-2-butane) and placed into the cryostat (2800 Frigocut, Reichert-Jung, Germany) until temperature equilibrated. Coronal sections (20μm) of the brain were cut (box and object temperature -25°C and -15°C respectively). Sections were mounted onto gelatine coated slides.

Animals that received i.c.v. colchicine were anaesthetised using 50mg kg^{-1} sodium pentobarbitone (Sagatal®) and mounted into a stereotaxic frame. The colchicine (75μg $20\mu l^{-1}$ sterile saline) was injected into the lateral ventricle (co-ordinates 1.6mm lateral, 1.8mm dorsal and 6.06mm anterior to inter-aural line zero), (König and Klippel 1974) over a period of 15 minutes. The scalp was sutured with silk thread. The animals were administered 100mg kg^{-1} ampicillin in 0.9% sterile saline (i.m.) to prevent infection following colchicine injection. The animals were sacrificed for CRF immunocytochemistry 48 hours following i.c.v. colchicine injection.

Gelatine-coated slides.

Slides were washed in hot soapy water, distilled water and finally rinsed with ethanol. 1g gelatine was dissolved in 100ml distilled water, heated to 50°C and 0.1g chromalin ($CrK(SO_4)_2.12H_2O$) added. The slides were immersed into the gelatine/chromalin solution and left to dry.

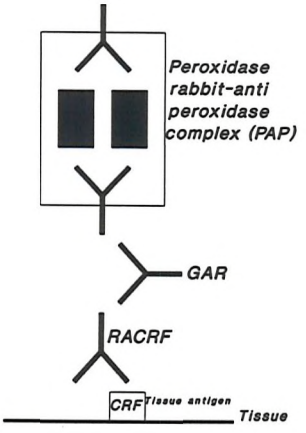
CRF immunocytochemistry.

The mounted tissue sections (5 per slide) were washed (3x10 minutes) with 0.9% NaCl buffered with 0.02M Tris-HCl at pH 7.6 (TBS), and then reacted with the primary CRF antiserum (RACRF) diluted 1:1000 with TBS containing 0.1% Triton X-100 (100µl slice^{-1}). The anti-CRF antibody was incubated for 24 hours at 4°C in a humidity chamber. The slides were then washed (3 x 10 minutes) and 100µl slice^{-1} of the secondary goat anti rabbit (GAR) antibody (diluted 1:40 with TBS containing 0.1% Triton X-100) was added to the slides and incubated for 2 hours at room temperature in a humidity chamber. The slides were washed again (three x 10 minutes) and the rabbit peroxidase-anti-peroxidase conjugate, (PAP), (diluted 1:40 with TBS containing 0.1% Triton X-100) added (100µl slice^{-1}) and incubated for 1 hour at room temperature in a humidity chamber. The sections were then washed (3 x 10 minutes). The DAB/H_2O_2 mix was prepared using 100mg DAB dissolved into 100ml 0.1M Tris-HCl (12.1g l^{-1}) added to 100ml 0.02% H_2O_2 (*v/v*), prepared in distilled water (60µl 30% H_2O_2 100ml water), immediately before use. The slides were left in the DAB/H_2O_2 solution for 4-6 minutes (until staining had developed), rinsed in TBS then distilled water and dehydrated through 50% (*v/v*), 70% (*v/v*), 90% (*v/v*) and absolute alcohol, and finally xylene. The slides were then mounted with DPX (BDH) and covered with glass coverslips.

Analysis of sections was performed using a Leitz orthoplan microscope (Ernst Leitz Wetzlar GMGB, Midland, Ontario, Canada) and appropriate photographs taken using Kodak Ektachrome 100 reverse colour film.

No staining was seen in rabbit anti-(rat/human)-CRF_{1-41} (1:1000) preabsorbed with 1nmole CRF_{1-41} ml^{-1} (5µg ml^{-1}). Abolition of positive staining was not seen with preincubation of rabbit anti-(rat/human)-CRF_{1-41} (1:1000) with neurotensin, CCK_8, bombesin, TRH, substance P, met-ENK or cGRP. Preabsorption of antibody was carried out by incubation with respective peptides at 4°C for 24 hours.

Figure 2.6. Diagramatic representation of (anti-CRF)-GAR-PAP complex.



2.7.8. IRMA for brain rat/human CRF_{1-41}.

The immunoradiometric assay for CRF_{1-41}, based on the use of radioisotopically labelled specific antibodies, has advantages over a RIA in that it is more sensitive, quicker and easier to perform. This IRMA uses an antibody directed against the N-terminal CRF fragment, CRF_{1-20}-NH_2, raised in guinea pigs (see section 2.1.3) and a second directed against the C-terminal fragment, CRF_{36-41}, raised in rabbits (see section 2.1.3), which were affinity-purified and radioiodinated (see Linton and Lowry 1986). Both antibodies were added together with standards or samples, and after incubation, separation of the guinea pig antiserum was accomplished using a sheep anti-(guinea pig Fc region) immunoglobulin (SAGp Fc IgG) solidphase. Radioactive rabbit anti-CRF_{36-41} is only precipitated in samples containing CRF_{1-41}, since the peptide acts as a link between the ^{125}I-labelled rabbit IgG and the unlabelled guinea pig CRF_{1-20} specific antibodies. The counts precipitated (bound) is directly proportional to the concentration of CRF_{1-41} in the added sample or standard.

Tissue preparation.

Rats (170g female Wistar) were killed by decapitation, the brain was immediately removed and placed into ice cold PBS for 2 minutes. The hypothalamus together with the medien eminence (ME) and pituitary stalk were removed (see section 2.7.3. for

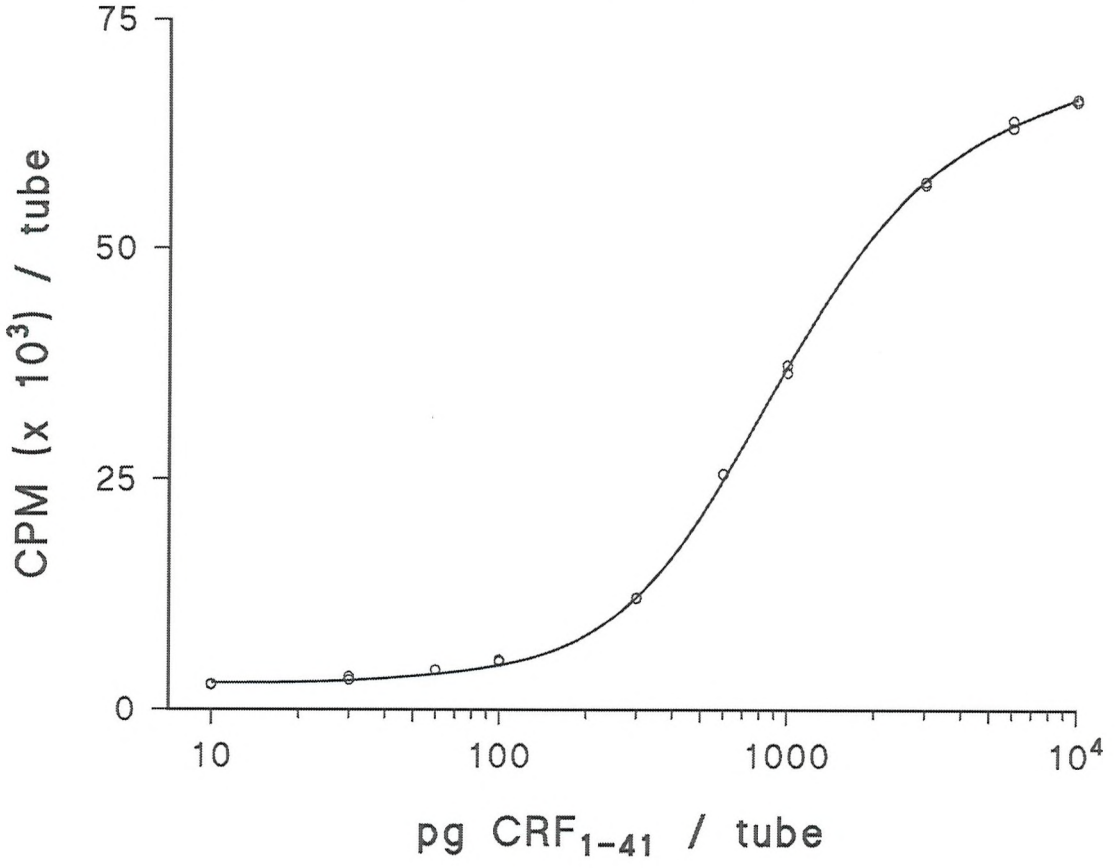
hypothalamus dissection), weighed to the nearest mg, homogenised in 1ml 10mM HCl containing 5.5mM ascorbic acid, heat-treated to destroy degrading enzymes (90°C for 10 minutes). The homogenates were then centrifuged (3000g at 4°C for 20 minutes), (Sorvall RT 7000 bench centrifuge), and the supernatants stored at -20°C until analysis.

IRMA protocol.

Standards or aliquots of samples were neutralised with NaOH, diluted in 200µl assay buffer (0.05M sodium phosphate containing 0.25% human serum albumin (Lister Institute, Elstree), 0.05% Triton X-100 and 0.01% sodium azide) and incubated for 16 hours at room temperature with 200µl of the CRF_{41} IRMA reagent mixture. This mixture consists of ^{125}I-labelled rabbit anti-(CRF_{36-41}) IgG (100000 cpm 200µl^{-1}), (see Linton and Lowry 1986) and guinea pig-anti-CRF_{1-20} serum (1:5000) in 0.05M sodium phosphate buffer pH 7.4, containing 0.05% human serum albumin (*w/v*) 10% normal rabbit serum (*v/v*) 0.01% and sodium azide (*w/v*). Separation of CRF-bound from free labelled IgG was achieved using SAGp Fc IgG coupled to Dynospheres (XP-6501, Dyno Industrier AS).

For the separation of the CRF-bound from the free labelled IgG, 100µl of resuspended SAGp Fc Dynospheres (prepared as in Linton and Lowry 1986) were mixed with the assay reagents and incubated for 30 minutes at room temperature. 1ml 0.01M $NaHCO_3$ containing 0.15M NaCl and 0.01% Triton X-100 was added, mixed and centrifuged (3000g for 20 minutes at 4°C). The supernatant was discarded and the pellets washed with 2ml of 0.01M $NaHCO_3$ buffer containing 0.15M NaCl and 0.01% Triton X-100, recentrifuged, and the radioactivity of the Dynosphere pellet measured in a gamma counter. A plot of radioactivity against log CRF_{1-41} per tube was plotted and concentrations of unknowns read off. A typical standard curve is shown in figure 2.7.

Figure 2.7. CRF IRMA standard curve.



2.7.9. GC analysis of brain and serum D-Fenfluramine and D-Nor-Fenfluramine.

Rat serum and brain samples were analysed for levels of D-Nor-Fen and D-Fen using a sensitive gas chromatographic system modified from Richards et al. 1989.

Preparation of samples.

The remainder of the brain to be analysed (section 2.5.), following the dissection of the hypothalamus and one hippocampus, was weighed to the nearest mg and homogenised in 6mls 0.5M H_2SO_4. The homogenate was then centrifuged (10 minutes at 3000g) and the supernatant used for analysis. Serum was used neat. Standards and quality control samples (brain and serum) were prepared in control brain homogenate and control rat serum.

Extraction and GC analysis.

100µl of standard (25, 50, 250, 500 or 1000ng ml^{-1}), quality controls (50, 125 or 1000ng ml^{-1}) or sample were added to tubes. 100µl of diethylaniline (DEA) internal standard (500ng ml^{-1}), 100µl 5M NaOH and 75µl butyl acetate were added, the tubes were mixed vigorously on a IKA-Vibrax-VXR vortex mixer for 5 minutes and then centrifuged for 15 minutes (2000g). The top (butyl acetate) phase was transferred into tapered sample microvials for automatic injection (3µl) onto the gas chromatograph.

GC system.

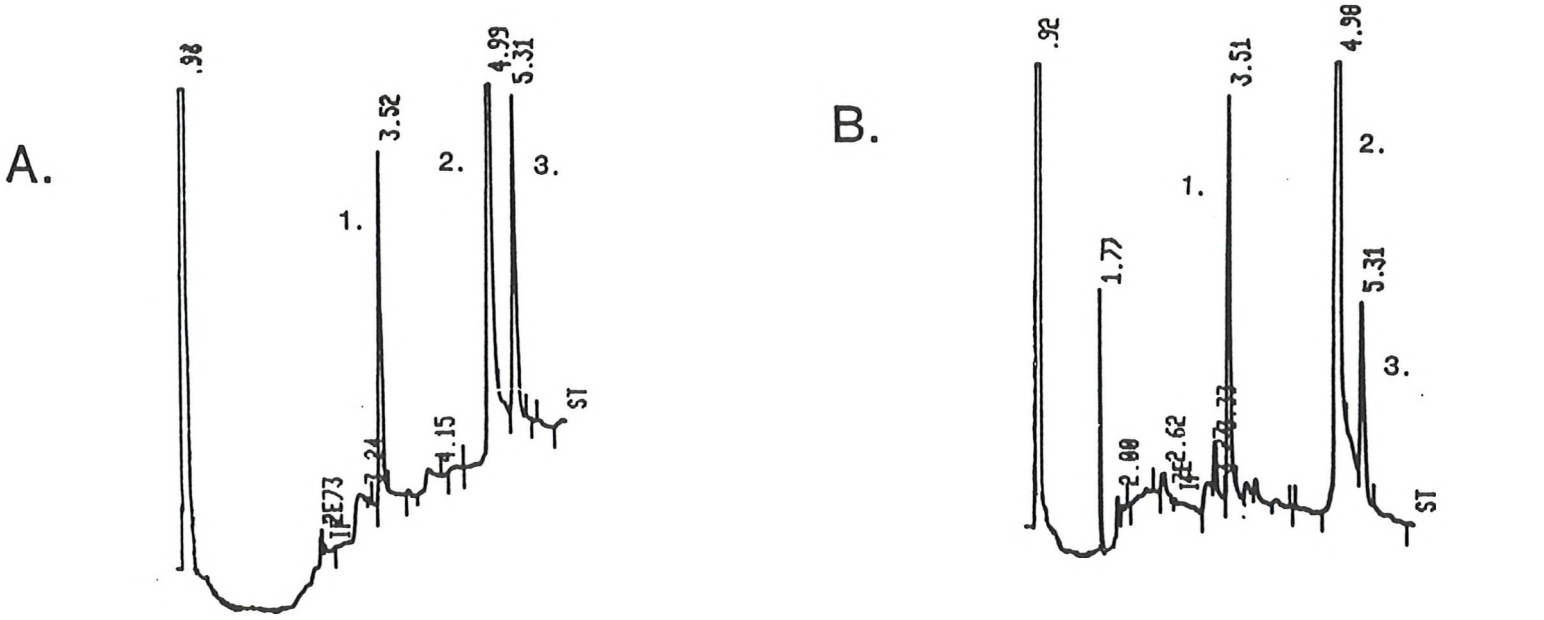
The GC system consists of a glass column (1.9mm x 2mm i.d.) packed with 10% Apeizon L and 10% potassium hydroxideon GasChrom Q support (100-200 mesh) mounted in a Hewlett-packard Model 5880 gas chromatograph and a constant flow (30ml min^{-1}) of oxygen-free nitrogen carrier gas. Injection block temperature 250°C, detector block temperature 300°C and oven temperature 120°C (isothermal). 3µl of injection volume was made with a Hewlett-Packard Model 7672A autosampler. The separated products were detected with a nitrogen-specific detector, with hydrogen and airflows of 3ml min^{-1} and 60ml min^{-1} respectively. Typical D-Nor-Fen, DEA and D-Fen elution times were 3.5, 5.0 and 5.3 minutes respectively. Peaks were integrated and corrected for losses by the correlation to the levels of internal standard. Coefficient of variations for D-Fen and D-Nor-Fen respectively ranged from 8.4% and 6.2%, 11.6% and 7.4%, 10.4% and 9.2%, 11.2% and 9.6%, 10.5% and 8.0% at 1039, 519, 260, 52 and 26ng ml^{-1} respectively in rat brain homogenate samples. Coefficient of variations for D-Fen and D-Nor-Fen respectively ranged from 8.4% and 6.2%, 11.6% and 7.4%, 10.4% and 9.2%, 11.2% and 9.6%, 10.5% and 8.0% at 1039, 519, 260, 52 and 26ng ml^{-1} respectively in rat serum samples.

Typical serum and rat brain homogenate GC traces are shown in figures 2.8. and 2.9. respectively.

Figure 2.8.

Typical GC chromatographs of:

- **A. 3µl injection rat serum extract standard containing 256ng ml^{-1} D-Nor-Fen (peak 1), 500ng ml^{-1} DEA internal standard (peak 2) and 260ng ml^{-1} D-Fen (peak 3).**
- **B. 3µl injection rat serum extract.**



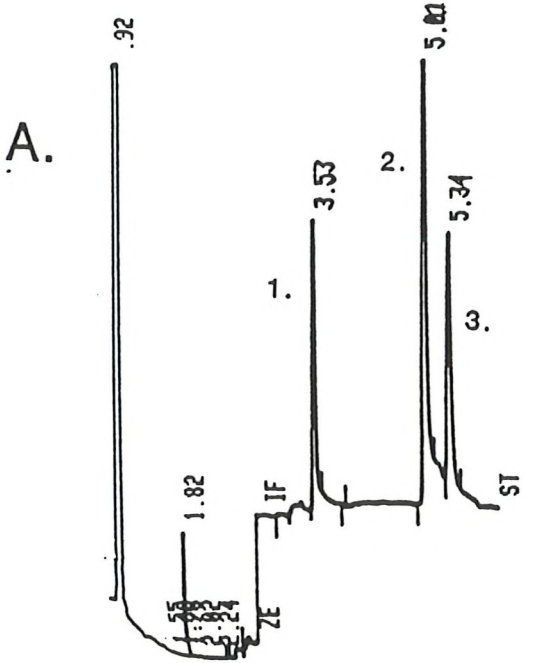
RT	HEIGHT	TYPE	CAL #	AMOUNT
3.52	20447	PB	1	0.325
4.99	62984	PB	2&	1.000
5.31	18696	BB	3	0.297

RT	HEIGHT	TYPE	CAL #	AMOUNT
3.51	23546	PB	1	0.288
4.98	81717	PB	2&	1.000
5.31	10498	BB	3	0.129

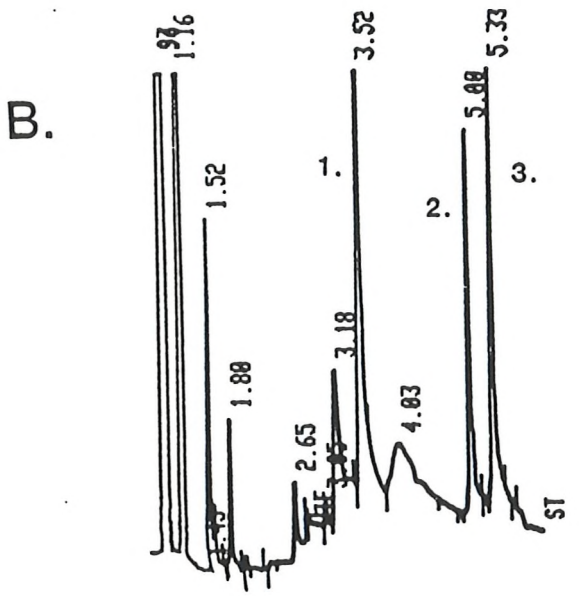
Figure 2.9.

Typical GC chromatographs of:

- **A.** **3µl injection rat serum extract standard containing 256ng ml^{-1} D-Nor-Fen (peak 1), 500ng ml^{-1} DEA internal standard (peak 2) and 260ng ml^{-1} D-Fen (peak 3).**
- **B.** **3µl injection rat brain extract.**



RT	HEIGHT	TYPE	CAL #	AMOUNT
3.53	16207	PB	1	0.436
5.01	37176	PB	2&	1.000
5.34	14267	BB	3	0.384



ISTD RT	HEIGHT	TYPE	CAL #	AMOUNT
3.52	69157	PB	1	3.188
5.00	21696	PB	2&	1.000
5.33	32338	PB	3 .	1.491

Chapter 3.

Results.

The paper by Arase *et al.* (1989c) demonstrated that adrenalectomy, in female Sprague-Dawley rats, prevented the restoration of normal food intake observed with D,L-Fenfluramine and increased body weight loss and GDP binding to interscapular brown adipose tissue. The experimental design by Arase *et al.* (1989c) was such that the animals were sacrificed 8 days following adrenalectomy.

I discovered that repeating the experiment of Arase *et al.* (1989c), with D-Fenfluramine in Wistar rats, but allowing the animals to survive beyond 8 days, resulted in the reversal of these effects.

In view of the apparent adaptation to the synergistic effects of D-Fen and adrenalectomy, it seemed logical to study possible loci and a mechanism for the interaction by investigation of the pharmacological and physiological systems associated with the modulation of D-Fen and adrenalectomy reduction in body weight.

The systems below were studied to show the interaction of D-Fenfluramine and adrenalectomy:

- The hypothalamic and hippocampal serotoninergic systems with a view to investigate if the actions of D-Fen or adrenalectomy singularly on these central 5-HT systems were modified by the combination of treatments.

- Body composition was studied with a view to understanding the relationship between anorexia and body set point and any differential effects on protein and lipid mobilisation, serum glucose and insulin and/or the efficiency of food deposition following the combination of D-Fen and adrenalectomy compared to either treatment alone.

- The hypothalamic CRF profile with a view in light of the previously demonstrated (by other investigators) rise in hypothalamic CRF levels following adrenalectomy, the effects of CRF on food intake and body weight and the stimulation of CRF release by D-Fenfluramine (and 5-HT).

- The metabolism of D-Fenfluramine and subsequent elimination of D-Fenfluramine and the primary metabolite of D-Fenfluramine, D-Nor-Fenfluramine was investigated to establish if the removal of glucocorticoids, by adrenalectomy, modified the brain and/or serum pharmacokinetic profile of D-Fenfluramine and/or D-Nor-Fenfluramine.

The animals were divided into groups allowed to recover (48 days following surgery, sacrifice day 51) and another sacrificed before this recovery (4 days following surgery, sacrifice 7).

In addition, confirmation of the adrenal factor responsible for the potentiation of the effects of D-Fen was investigated by replacement corticosterone therapy to adrenalectomised ± D-Fen animals.

The culmination of this investigation is presented in the following six sections:

Chapter 3., Section 3.1.

- Effect of adrenalectomy in acute and chronic D-Fenfluramine treated female Wistar rats on body weight and food intake profiles, serum glucose and insulin, liver weight and ovarian fat pad weights.

Chapter 3., Section 3.2.

- Effect of adrenalectomy in acute and chronic D-Fenfluramine treated female Wistar rats on the hypothalamic and hippocampal serotoninergic system.

Chapter 3., Section 3.3.

- Effect of adrenalectomy in acute and chronic D-Fenfluramine treated female Wistar rats on body composition and the metabolic fate of energy intake.

Chapter 3., Section 3.4.

- Effect of corticosterone-replacement on the response to adrenalectomy in acute D-Fenfluramine treated female Wistar rats.

Chapter 3., Section 3.5.

- Effect of adrenalectomy in acute D-Fenfluramine treated female Wistar rats on hypothalamic corticotrophin-releasing factor (CRF).

Chapter 3., Section 3.6.

- Effect of adrenalectomy in acute and chronic D-Fenfluramine treatment, and corticosterone-replacement to acute D-Fenfluramine treated adrenalectomised female Wistar rats on the brain and serum levels of D-Fen and its primary metabolite, D-Nor-Fen.

The term acute and chronic D-Fen treatment is applied to the two basic experimental designs. Acute D-Fen treatment is applied to those animals sacrificed on day 7 (Section 3.1-3) or day 8 (section 3.4) whilst chronic treatment is applied to those animals allowed to continue beyond the period demonstrated by Arase *et al.* (1989c) and sacrificed on day 51.

3.1. Effect of adrenalectomy in acute and chronic D-Fenfluramine treated female Wistar rats on body weight and food intake profiles, serum glucose and insulin, liver weight and ovarian fat pad weights.

3.1.1.

In view of the apparent adaptation to the synergistic effects of D-Fen and adrenalectomy, the animals were divided into groups allowed to recover (48 days following surgery, sacrifice day 51) and another sacrificed before this recovery (4 days following surgery, sacrifice 7).

The experimental methods are listed in Chapter 2.

Six week old female Wistar rats were individually housed for an initial seven days to allow acclimatisation to handling and the environment. During this period body weights and food intake were monitored daily. Nine groups (n=6) were used.

- Group 1 was sacrificed on day 1, (termed the day 1 controls).

The remaining 8 groups were divided thus:

- Four receiving daily (p.m.) injections of sterile isotonic saline (1ml kg^{-1}) and
- Four groups receiving daily (p.m.) injections of D-Fen (10mg kg^{-1}) in sterile saline (10mg ml^{-1}).

The first day of D-Fen or saline treatment was termed day 1. The body weights and previous 24 hours food intake were measured between 0900-1000 hours each day.

On day 3 the animals were bilaterally adrenalectomised or sham adrenalectomised (see Chapter 2 for protocol) to provide:

- Two groups of sham-ADX-operated animals receiving D-Fen (sham-ADX D-Fen),
- Two groups of bilaterally adrenalectomised animals receiving D-Fen (ADX D-Fen),
- Two groups of sham-ADX-operated animals receiving saline (sham-ADX saline) and
- Two groups of bilaterally adrenalectomised animals receiving saline (ADX saline).

One each of the experimental groups were maintained until day 7, and were then sacrificed, whilst the remaining four corresponding experimental groups were allowed to proceed until day 51 (see Chapter 2 for termination details). The table below summarises the animal groups and their respective sacrifice days.

Group	Treatment	Day of Sacrifice
1	None (Day 1 control)	1
2	Sham-ADX D-Fen	7
3	ADX D-Fen	7
4	Sham-ADX saline	7
5	ADX saline	7
6	Sham-ADX D-Fen	51
7	ADX Fen	51
8	Sham-ADX saline	51
9	ADX saline	51

3.1.2 Body weight profiles for acute treatment.

The body weight profiles of animals sacrificed on day 7 are presented in Figure 3.1. The body weights of both D-Fen-treated animals demonstrated a fall that was maintained at a level below that of the two other saline-treated groups (sham-ADX saline and ADX saline), until day 3 when surgery was performed.

Figure 3.1. The effect of acute D-Fen and adrenalectomy on the change in body weight.

The following groups were studied; sham-ADX saline (open square), ADX saline (open triangle), sham-ADX D-Fen (cross) and ADX D-Fen (open circle).

Data points are means ± SEM. All data groups contain six animals. The solid bar represents the period of daily D-Fen injections (10mg kg^{-1} day^{-1}) in sterile saline (10mg ml^{-1}) or sterile saline vehicle (1ml kg^{-1}) alone between 1700 and 1800 hours (i.p.).

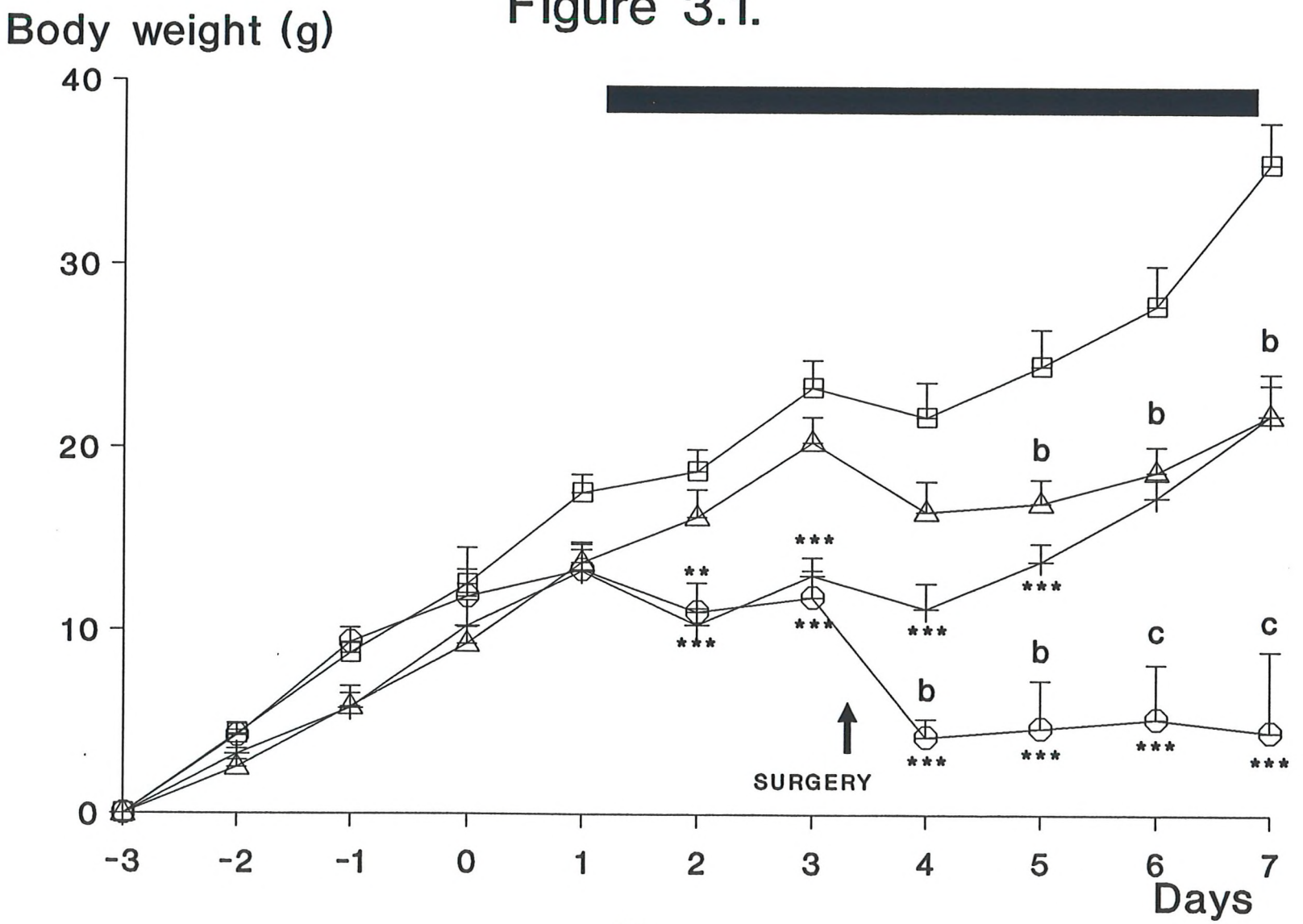
2 way ANOVA shows significant effect of adrenalectomy versus sham-ADX surgery on days 4 to 7 with a p value of <0.001 for all days. See appendix 1 for F values.

2 way ANOVA shows significant effect of D-Fen versus saline on days 2 to 7 with a p value of <0.001 for all days. See appendix 1 for F values.

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

Figure 3.1.



The sham-ADX saline group (open squares) showed a slight fall in body weight associated with surgery, but body weight was restored to a normal growth pattern by day 4. The ADX saline-treated animals displayed the usual anorexia, following adrenalectomy, with a maintained body weight significantly below that of the sham-ADX saline group ($p<0.01$). The body weight of sham-ADX D-Fen-treated animals fell following surgery but recovered at a similar rate to the ADX saline animals. The body weight of the ADX saline animals was maintained at a significantly lower level to that of the sham-ADX saline animals ($p<0.05$), and was non- significantly different from sham-ADX D-Fen treated animals. The body weight of the ADX D-Fen-treated animals fell approximately 10g, following surgery, and then remained stable until the end of the study (day 7).

The ADX D-Fen group had significantly reduced body weights compared to all groups ($p<0.001$ to all groups). A significant effect of surgery and D-Fen treatment, by 2 way ANOVA, was seen for days 4 to 7 and 2 to 7 respectively ($p<0.001$ for all days). See appendix for F values.

3.1.3 Food intake profiles for acute treatment.

The 24 hour food intake profiles of the animals in Figure 3.1. are presented in Figure 3.2. The food intake profiles reflected the responses in body weight. D-Fen administration produced an approximate 60% reduction in food intake on day 1, but food intake returned towards control levels (at approximately 75% control levels) on day 2. Surgery, on day 3, produced a fall in food intake for all groups with food intake of sham-ADX saline>sham-ADX D-Fen>ADX saline>ADX D-Fen. The food intake for the sham-ADX D-Fen animals was restored to control sham-ADX saline levels by day 4, and remained so until termination on day 7. The food intake of sham-ADX D-Fen rats was non-significantly different from sham-ADX saline rats from day 3 onwards except for day 5. The food intake of ADX saline rats remained significantly reduced compared to the sham-ADX saline groups from day 3 until termination ($p<0.001$).

Figure 3.2. The effect of acute D-Fen and adrenalectomy on daily food intake.

The following groups were studied; sham-ADX saline (open square), ADX saline (open triangle), sham-ADX D-Fen (cross) and ADX D-Fen (open circle).

Data points are means ± SEM. All data groups contain six animals. The solid bar represents the period of daily D-Fen injections (10mg kg^{-1} day^{-1}) in sterile saline (10mg ml^{-1}) or sterile saline vehicle (1ml kg^{-1}) alone between 1700 and 1800 hours (i.p.).

2 way ANOVA shows significant effect of adrenalectomy versus sham-ADX surgery on days 3 to 6 with a p value of $p<0.001$ for all days. See appendix 1 for F values.

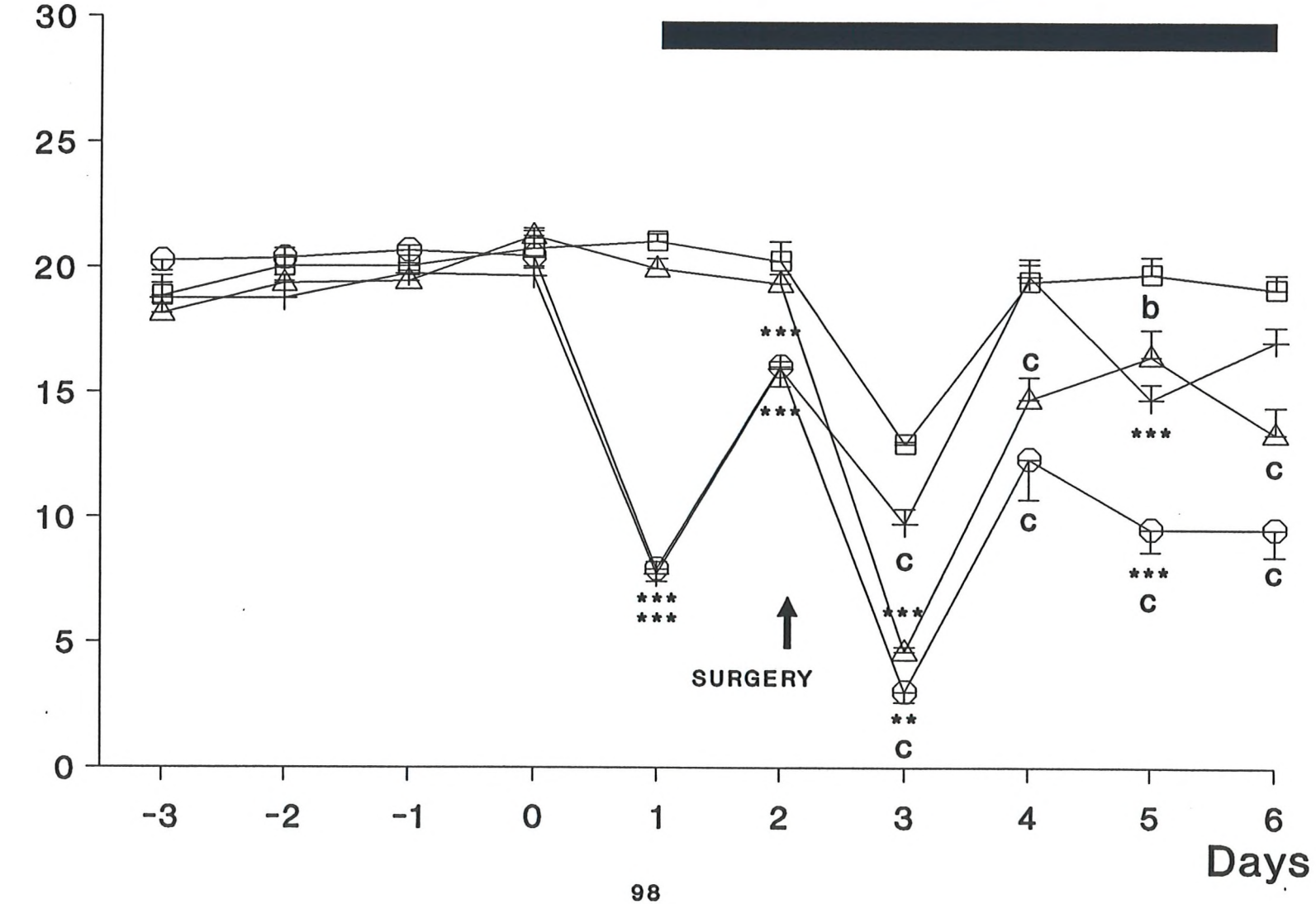
2 way ANOVA shows significant effect of D-Fen versus saline on days 1 to 3, 5 and 6 with p values of $p<0.001$, $p<0.001$ and $p=0.004$ respectively. See appendix 1 for F values.

Significant interaction between groups is seen on day 3 ($p=0.040$) $F(1,24)=4.8$.

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

Figure 3.2.


Daily food Intake (g)
30
25
20
15
10
5
0
-3
-2
-1
0
1
2
3
4
5
6
Days
SURGERY
***
***
***
***
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***
***
b
c
c
c
c
c
c
c

The ADX D-Fen animals showed a significant reduction in food intake which was maintained at approximately 50% control levels, and was significantly lower than all other groups until termination ($p<0.001$). Two way ANOVA revealed a significant effect of surgery and D-Fen treatment for days 3 to 7 and 2 to 7 respectively ($p<0.001$ for all days). See appendix for F values.

3.1.4. Body weight profiles for chronic treatment.

The body weight profiles of the animal groups that were allowed to continue until day 51 are presented in Figure 3.3. The body weight profiles in these animals, for the period up to day 7, were identical to the profiles of the acutely treated animals displayed in Figure 3.1., and therefore can be assumed to be an accurate reflection of the acute treatment animals if they were allowed to continue until day 51. The body weights of both ADX saline and sham-ADX D-Fen groups were maintained at levels below the sham-ADX saline animals until termination on day 51. Sham-ADX D-Fen body weights were significantly lower than sham-ADX saline animals from day 2 until termination. ADX saline animals body weights were not significantly lower than sham-ADX saline animals, but clearly were following a growth pattern maintained below that of the sham-ADX saline animals.

The body weight profile of the ADX D-Fen animals is clearly important. The anorexia was maintained until day 10, after which an accelerated recovery phase appeared for 7 days. This was followed by a more gradual recovery back to the sham-ADX D-Fen levels by the end of the study. ADX D-Fen animals had significantly lower body weights compared to sham-ADX D-Fen animals from day 5 until day 14 only. The body weights of these animals were significantly lower than the sham-ADX saline animals from day 2 until the end of the study.

Figure 3.3. The effect of chronic D-Fen and adrenalectomy on the change in body weight.

The following groups were studied; sham-ADX saline (open square), ADX saline (open triangle), sham-ADX D-Fen (cross) and ADX D-Fen (open circle).

Data points are means ± SEM. All data groups contain six animals. The solid bar represents the period of daily D-Fen injections (10mg kg^{-1} day^{-1}) in sterile saline (10mg ml^{-1}) or sterile saline vehicle (1ml kg^{-1}) alone between 1700 and 1800 hours (i.p.).

2 way ANOVA shows significant effect of adrenalectomy versus sham-ADX surgery on days 6 to 15, days 19 to 31 and days 37 to 44. See appendix 1 for F values.

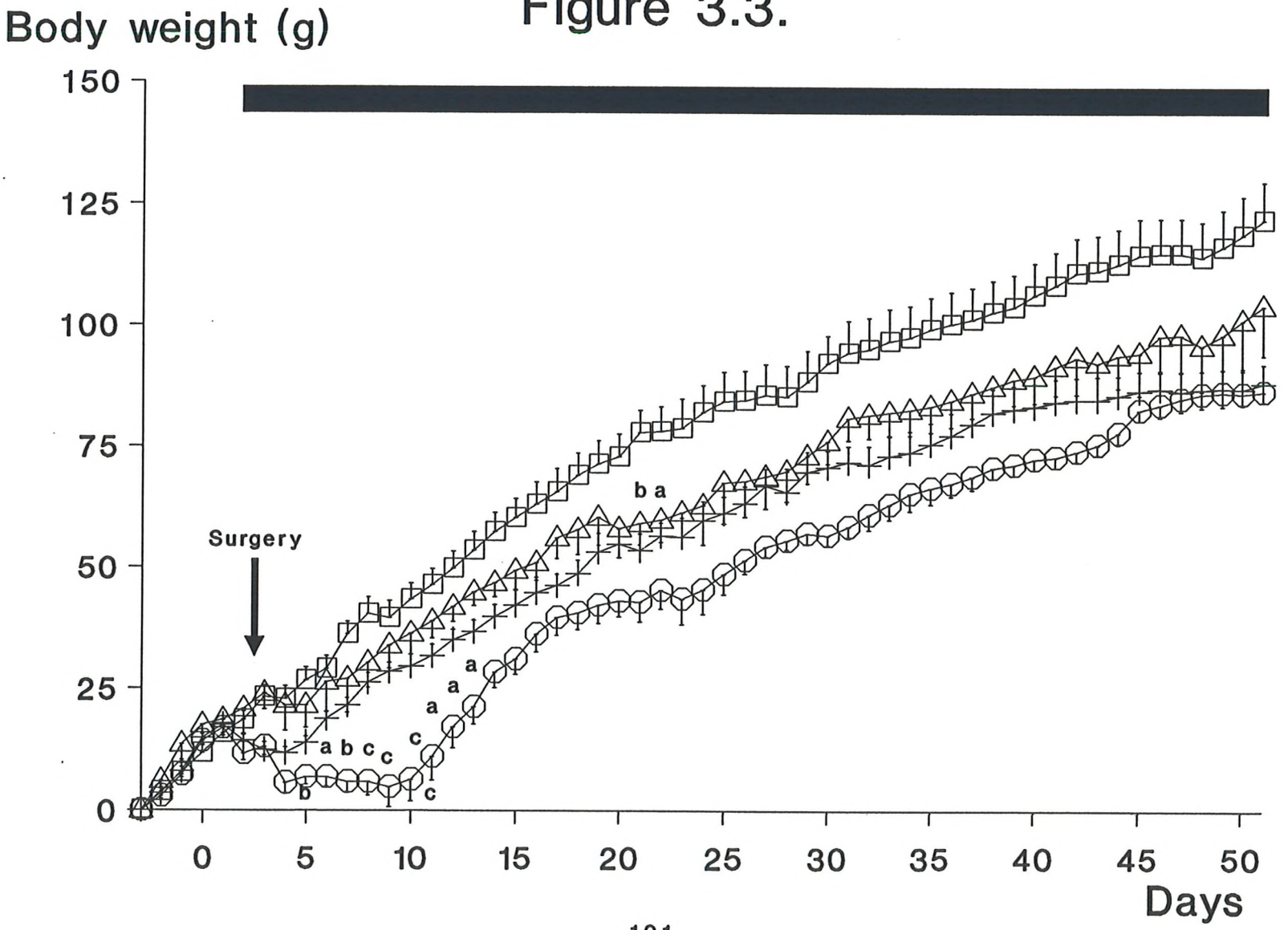
2 way ANOVA shows significant effect of D-Fen versus saline on days 2 to 51 with a p value of <0.001 for all days. See appendix 1 for F values.

Interaction between groups is seen on days 6 ($p=0.023$) $F(1,24)=6.2$, day 7 ($p=0.010$) $F(1,24)=8.4$, day 8 ($p=0.012$) $F(1,24)=7.8$, day 9 ($p=0.006$) $F(1,24)=10.0$ and day 10 ($p=0.011$) $F(1,24)=8.3$.

Student Newman-Keul's analysis shows significant differences sham-ADX saline and sham-ADX D-Fen on days 3 to 10 ($p<0.05$), 11 to 18 ($p<0.01$), 19 to 29 ($p<0.05$), 30 to 35 ($p<0.01$), 36 to 40 ($p<0.05$) and 41 to 51 ($p<0.01$). Significant differences between ADX saline and ADX D-Fen is seen on days 4 and 5 ($p<0.01$), 6 to 13 ($p<0.001$), 14 and 15 ($p<0.01$), 16 to 21 ($p<0.05$), 30 to 33 and 39 to 42 ($p<0.05$).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

Figure 3.3.


Body weight (g)
150
125
100
75
50
25
0
Surgery
b a
a b c c
c
a
a
a
c
b
c
0
5
10
15
20
25
30
35
40
45
50
Days

3.1.5. Food intake profiles for chronic treatment.

The food intake profiles for the animals in Figure 3.3. are presented in Figures 3.4. and 3.5. The food intake profiles in these animals, for the period up to day 7, were identical to the profiles in Figure 3.2. This data further supports the assumption that there was no inherent difference between the acute and chronic treatment groups. The sham-ADX D-Fen animals food intake was significantly lower than the sham-ADX saline animals only for days 1 to 3, there being no statistically significant difference between the groups thereafter. The ADX saline animals showed a significant reduction in daily food intake from sham-ADX saline for day 3 to day 12, and periodically during the study some days had a significantly reduced intake. Food intake in the ADX D-Fen animals was significantly lowered compared to ADX saline on days 1 to 3 and 7 to 10, and significantly lower than sham-ADX D-Fen from day 3 until day 10, and was non-significantly different for the remainder of the study.

Figure 3.4. The effect of chronic D-Fen and adrenalectomy on daily food intake.

Part 1. The following groups were studied; sham-ADX saline (open square), ADX saline (open triangle), sham-ADX D-Fen (cross) and ADX D-Fen (open circle). This figure shows the D-Fenfluramine treated animals.

Data points are means ± SEM. All data groups contain six animals. The solid bar represents the period of daily D-Fen injections (10mg kg^{-1} day^{-1}) in sterile saline (10mg ml^{-1}) between 1700 and 1800 hours (i.p.).

2 way ANOVA shows significant effect of adrenalectomy versus sham-ADX surgery on days 3 to 10, day 20 and 23 with p values of $p<0.001$, $p=0.026$ and $p=0.010$ respectively. See appendix 1 for F values.

2 way ANOVA shows significant effect of D-Fen versus saline on days 1 to 3, 5, 8, 9, 12, 13, 23 with p values of $p<0.001$, $p=0.014$, $p=0.035$, $p=0.005$, $p=0.007$, $p=0.046$ and $p=0.016$ respectively. See appendix 1 for F values.

Student Newman-Keul's analysis shows significant differences sham-ADX saline and sham-ADX D-Fen on days 1 and 3 ($p<0.001$) and 2 ($p<0.01$). Significant differences between ADX saline and ADX D-Fen are seen on days 1 ($p<0.001$), 2 and 3 ($p<0.01$) and 7 to 10 ($p<0.05$).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

Figure 3.4.

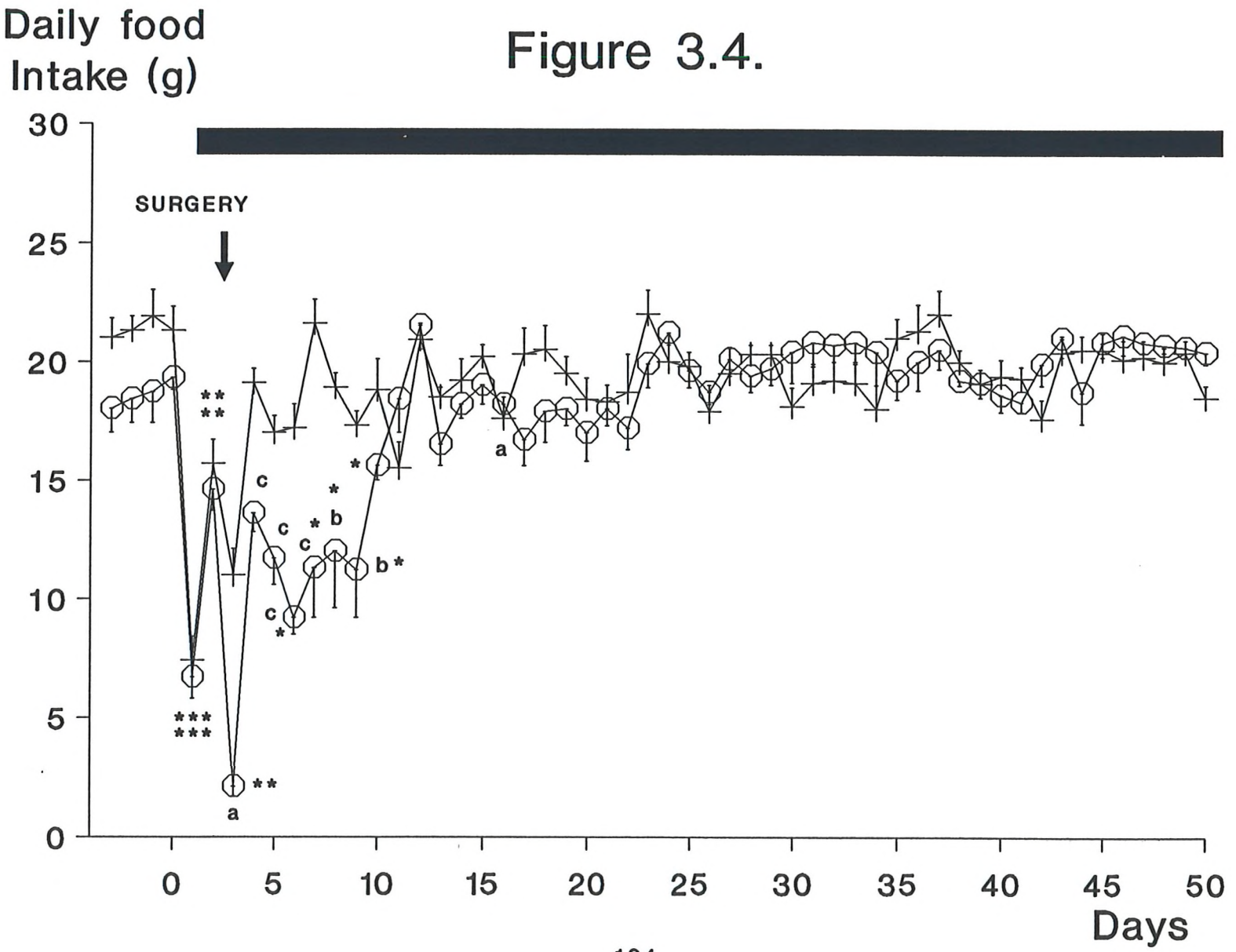

Daily food Intake (g)
30
25
20
15
10
5
0
SURGERY
0
5
10
15
20
25
30
35
40
45
50
Days

Figure 3.5. The effect of chronic D-Fen and adrenalectomy on daily food intake.

Part 2. The following groups were studied; sham-ADX saline (open square), ADX saline (open triangle), sham-ADX D-Fen (cross) and ADX D-Fen (open circle). This figure shows the saline treated animals.

Data points are means ± SEM. All data groups contain six animals. The solid bar represents the period of daily sterile saline vehicle injections (1ml kg^{-1}) between 1700 and 1800 hours (i.p.).

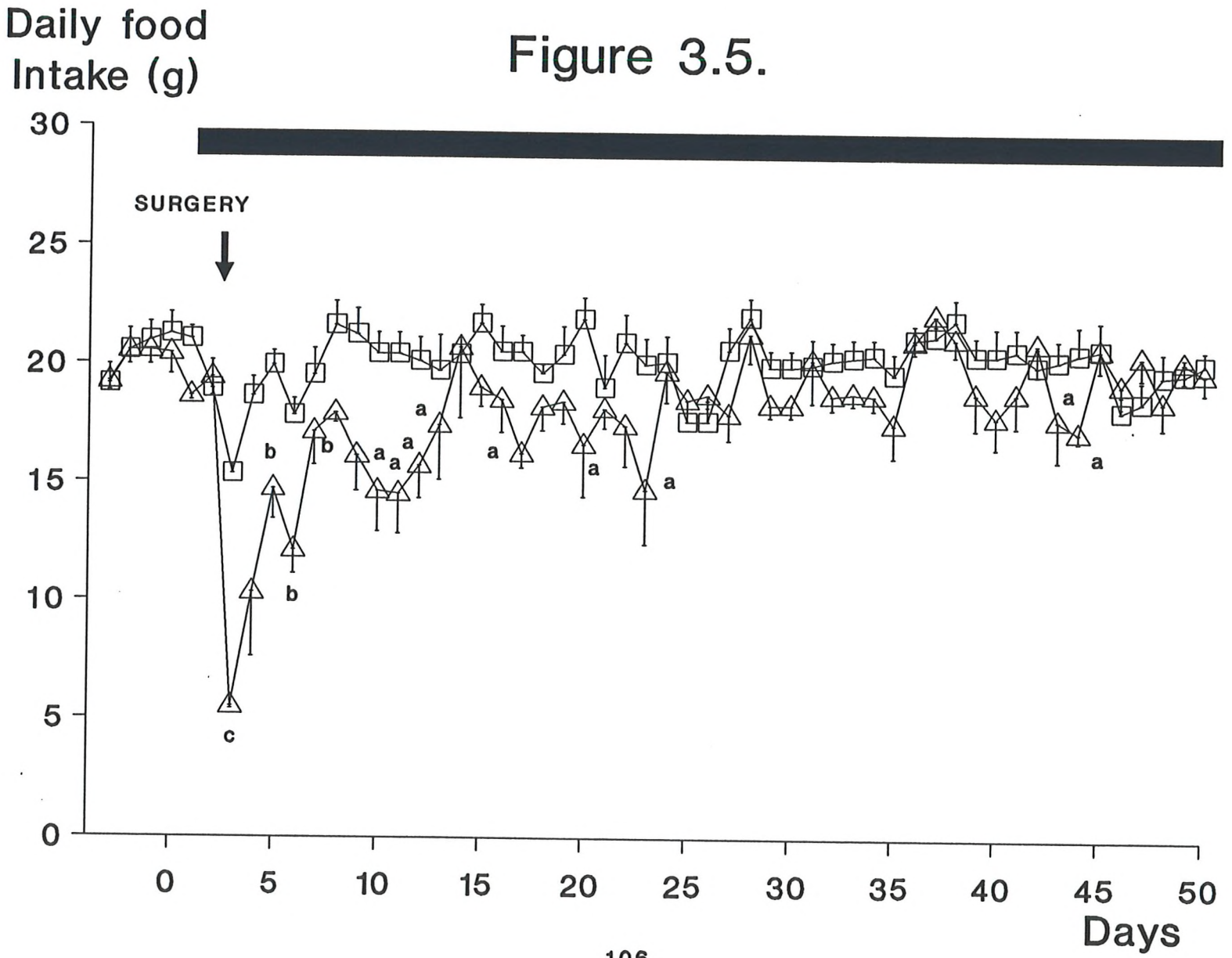
2 way ANOVA shows significant effect of adrenalectomy versus sham-ADX surgery on days 3 to 10, day 20 and 23 with p values of $p<0.001$, $p=0.026$ and $p=0.010$ respectively. See appendix 1 for F values.

2 way ANOVA shows significant effect of D-Fen versus saline on days 1 to 3, 5, 8, 9, 12, 13, 23 with p values of $p<0.001$, $p=0.014$, $p=0.035$, $p=0.005$, $p=0.007$, $p=0.046$ and $p=0.016$ respectively. See appendix 1 for F values.

Student Newman-Keul's analysis shows significant differences sham-ADX saline and sham-ADX D-Fen on days 1 and 3 ($p<0.001$) and 2 ($p<0.01$). Significant differences between ADX saline and ADX D-Fen is seen on days 1 ($p<0.001$), 2 and 3 ($p<0.01$) and 7 to 10 ($p<0.05$).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

Figure 3.5.


Daily food Intake (g)
30
25
20
15
10
5
0
SURGERY
a
b
b
a
a
a
a
a
a
a
a
b
c
0
5
10
15
20
25
30
35
40
45
50
Days

3.1.6. Serum glucose and insulin levels, liver and ovarian fat masses for acute and chronic treatment.

Serum levels of glucose and insulin, and liver and ovarian fat masses, of the animals from Figures 3.1. to 3.5. are presented in Table 3.1.

In the chronic ADX D-Fen treated group, liver and ovarian fat masses increased from day 7. These changes were in line with the body weight recovery profiles exhibited in Figure 3.3. By day 51 all liver weights were non-significantly different to each other, and the ovarian fat masses in both adrenalectomised groups (ADX saline and ADX D-Fen) were non-significantly different but still significantly reduced compared to both sham (sham-ADX saline and sham-ADX D-Fen) animal groups.

Serum insulin levels were significantly reduced in both adrenalectomised animal groups, compared to both sham-ADX groups, sacrificed on day 7 and day 51 ($p<0.001$ and $p=0.008$ respectively). No significant effect, by 2 way ANOVA, of D-Fen versus saline was seen. Serum glucose was significantly reduced in adrenalectomy versus sham-ADX animals for both day 7 and day 51 ($p=0.03$ and $p=0.040$). No significant effect, by 2 way ANOVA, of D-Fen versus saline was seen.

Table 3.1. The effect of acute and chronic D-Fen and adrenalectomy on serum glucose (mM), insulin (μU ml^{-1}), liver weights (g) and ovarian fat mass weight (g) for the animal groups depicted in Figures 3.1. to 3.5 (Section 3.2).

Data points are means ± SEM. All data groups contain six animals.

See appendix 2 for ANOVA F values (reference number in parentheses).

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively) for respective sacrifice days.

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively) for respective sacrifice days.

Table 3.1.

	Serum Glucose (mM)		Serum Insulin ($\mu U\ ml^{-1}$)		Liver weight (g)		Ovarian Fat Pad Weight (g)	
Treatment	Day 7	Day 51	Day 7	Day 51	Day 7	Day 51	Day 7	Day 51
Sham D-Fen	5.83 ± 0.17	6.83 ± 0.17	84.2 ± 11.4	62.9 ± 6.9	6.07 ± 0.35 *	6.76 ± 0.31	0.30 ± 0.04	2.33 ± 0.39
ADX D-Fen	5.15 ± 0.19 b	6.61 ± 0.28	37.2 ± 4.6 c	45.2 ± 7.0	4.28 ± 0.47 * b	6.08 ± 0.52	0.06 ± 0.02 b	0.89 ± 0.12 b
Sham Saline	5.93 ± 0.08	7.33 ± 0.11	71.8 ± 9.2	72.0 ± 8.6	7.26 ± 0.38	7.25 ± 0.41	0.41 ± 0.07	2.92 ± 0.29
ADX Saline	5.52 ± 0.11	6.75 ± 0.18 a	43.2 ± 5.4 a	38.4 ± 9.6 a	5.90 ± 0.44 a	5.98 ± 0.29	0.15 ± 0.02 c	0.77 ± 0.18 c
2 Way Anova								
Sham v ADX	p=0.003 (1)	p=0.046 (4)	p<0.001(7)	p=0.008 (10)	p=0.001 (13)	p=0.030 (16)	p<0.001 (19)	p<0.001 (22)
D-Fen v saline	NS (2)	NS (5)	NS (8)	NS (11)	p=0.003 (14)	NS (17)	p=0.024 (20)	NS (23)
Interaction	NS (3)	NS (6)	NS (9)	NS (12)	NS (15)	NS (18)	NS (21)	NS (24)
Day 1 Control	5.35 ± 0.11		37.5 ± 18.8		4.40 ± 0.32		0.20 ± 0.02	

3.1.7. Total food intake and change in body weight during acute and chronic studies of D-Fen and adrenalectomy.

The cumulative food intake and the change in body weight for the animals sacrificed on day 7 are presented in Table 3.2. The cumulative food intake and the change in body weight for days 1 to 7 and days 1 to 51 are presented in Table 3.3. The total food intake and body weight changes, of ADX D-Fen animals, from day 1 to 7 show significant reductions compared to all other groups ($p<0.001$ in both cases), with significance, by two way ANOVA, of both surgery and D-Fen ($p<0.001$ in both cases), with a significant interaction between adrenalectomy and D-Fen ($p=0.016$ and $p=0.024$ respectively). This trend was duplicated in the total food intake and body weight changes for the period day 1 to 7 in the animals allowed to continue to day 51, with the effect of adrenalectomy and D-Fen at day 7 for these animals significantly reducing cumulative food intake ($p<0.001$ in both cases) and producing a significant interaction ($p=0.041$ and $p=0.011$ respectively). This further confirms the fact that these animals can be assumed to be accurate reflections of the acute treatment animals if they were allowed to continue until day 51. By day 51 the effect of surgery, by two way ANOVA, is significant ($p=0.019$), whilst the effect of D-Fen was non-significant. The total food intake of ADX D-Fen can be seen to have been restored to the level of sham-ADX D-Fen and ADX saline. The change in body weight showed a significant effect, by two way ANOVA, of D-Fen versus saline ($p<0.001$), with the effect of surgery being non-significant.

3.1.8. Serum corticosterone at day 7 and 51 for acute and chronic treatment.

Serum corticosterone levels in all four adrenalectomised animals are below the limits of quantification ($<15 mg\ l^{-1}$).

The corticosterone values for the sham-ADX D-Fen and sham-ADX saline animals sacrificed on day 6 are 1659 ± 333 and 1450 ± 382 ng ml^{-1} respectively, with the animals sacrificed on day 51 displaying levels of 1837 ± 365 and 1605 ± 444 ng ml^{-1} respectively.

Table 3.2. The effect of acute D-Fen and adrenalectomy on total food intake and the change in body weight for the period from the initiation of D-Fen treatment (day 1) until termination on day 7 for the animal groups depicted in Figures 3.1. and 3.2 (section 3.1).

Table 3.3. The effect of chronic D-Fen and adrenalectomy on total food intake and the change in body weight for the period from the initiation of D-Fen treatment (day 1) until day 7 (A) and termination on day 51 (B) for the animal groups depicted in Figures 3.3., 3.4. and 3.5 (section 3.1).

Data points are means ± SEM. All data groups contain six animals.

See appendix 2 for ANOVA F values (reference number in parentheses).

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively) for respective sacrifice days.

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively) for respective sacrifice days.

Table 3.2.

Treatment	Σ Food Intake (g)	Change in Body Weight (Δg)
Sham D-Fen	84.5 ± 0.6 ***	9 ± 2 *
ADX D-Fen	57.9 ± 2.3 *** c	- 8 ± 4 *** c
Sham Saline	113.9 ± 2.0	18 ± 2
ADX Saline	88.2 ± 1.3 b	8 ± 2 b

2 Way Anova		
Sham v ADX	p<0.001 (1)	p<0.001 (4)
D-Fen v saline	p<0.001 (2)	p<0.001 (5)
Interaction	p=0.016 (3)	NS (6)

Table 3.3.

Treatment	(A) Σ Food Intake (g)	(B)Σ Food Intake (g)	(A) Change in BW (Δg)	(B) Change in BW (Δg)
Sham D-Fen	87.4 ± 2.9 ***	938 ± 23	4 ± 5 ***	72 ± 3 ***
ADX D-Fen	57.9 ± 2.3 *** c	873 ± 20	-11 ± 3 *** c	66 ± 6
Sham Saline	109.5 ± 3.7	1000 ± 38	20 ± 1	104 ± 6
ADX Saline	85.4 ± 3.2 c	909 ± 16	9 ± 4 b	86 ± 9

2 Way Anova				
Sham v ADX	p<0.001 (1)	p=0.019 (4)	p=0.009 (7)	P=0.07 (10)
D-Fen v saline	p<0.001 (2)	NS (5)	p=0.013 (8)	p<0.001 (11)
Interaction	p=0.041 (3)	NS (6)	p=0.011 (9)	NS (12)

Although the trend is for the sham-ADX D-Fen animals to have higher corticosterone levels for both days, all groups are non-significantly different from each other.

3.1.9. Discussion.

The body weight profile for sham-ADX D-Fen animals were typical of the body weight changes seen in free-feeding rodents and in humans administered with Fen or D-Fen (Innes *et al.* 1977, Duhault *et al.* 1979, Stunkard 1981, Douglas *et al.* 1983, Brindley *et al.* 1985, Rowland and Carlton 1986b). There was an initial weight loss relative to controls, followed by restoration of the normal growth rate but at a trajectory below that of controls (sham-ADX saline) of approximately 40g after 51 days of administration. This action is not thought to be a reflection of tolerance to the actions of D-Fen, but possibly to metabolic adaptations to the lower weight.

The recovery in food intake to control levels by 3 days after D-Fen initiation was typical of the food intake profiles demonstrated by other investigators (animals (Duhault *et al.* 1979, Brindley 1985, Fantino *et al.* 1986, 1988). Tolerance to the actions of Fen were observed within 2 to 5 days, using various feeding paradigms including food deprivation schedules (Antelman *et al.* 1981, Curtis-Prior and Prouteau 1983, Heffer and Seiden 1979, Goudie *et al.* 1974, Rowland *et al.* 1982), a dessert test (Carlton and Rowland 1984) or tail-pressure eating (Antelman *et al.* 1981). 5mg kg^{-1} of Fen initially suppressed dessert intake by 80%, with 50% and 20% suppression on days 2 and 3, and a non-significant effect thereafter (Carlton and Rowland 1984).

Adrenalectomy produces anorexia and an increase in energy expenditure, in rodents, by the transference of energy away from storage mechanisms to energy wastage by the mitochondria of brown adipose tissue (Holt and York 1982, 1983). Adrenalectomy produces an increase in BAT NA turnover and GDP binding to mitochondria in interscapular brown adipose tissue (Holt and York 1982, 1983). Treatment of adrenalectomised animals with corticosterone reduces GDP binding. The anorexia, in this study, was maintained at a body weight approximately 20g below sham-ADX controls.

The observation that adrenalectomy and D-Fen both produce weight loss prompted the hypothesis that combination of the two might produce enhanced weight loss. This was indeed the case, with adrenalectomised D-Fen treated female Wistar rats maintaining anorexia below that of either treatment alone. This data confirms the results obtained by Arase *et al.* (1989), using D,L-Fen, in the maintenance of a body weight approximately 10g below sham-ADX D-Fen animals 9 days following surgery. However, prolonging the experimental period produced a reversal in the body weight profile and anorexia seen by Arase *et al.* (1989c).

The normal feeding pattern, in rodents, is to eat during the dark cycle (1900-0700 hours), with minimal feeding during the light cycle. The restoration of feeding in ADX D-Fen animals at day 10 does not reflect a change of feeding to the light cycle when the levels of D-Fen and D-Nor-Fen would be expected to be lower. Rowland and Carlton (1989b) demonstrate that the time of injection of Fen made no difference between to feeding during the dark cycle.

Adrenalectomy reduced both serum insulin and glucose levels. The reduction in glucose levels by adrenalectomy may be attributable to a loss of hepatic-stimulated glucose production and the removal of glucocorticoid inhibition of glucose uptake. Insulin levels were decreased by the removal of glucocorticoid stimulation of PSNS-stimulation of insulin secretion. This underlines the importance of glucocorticoid effects on insulin secretion. It would be expected that a lowering of serum insulin would cause an increase in glucose levels not the reduction observed, suggesting the glucocorticoid effects on glucose production are greater than insulin secretion.

Liver and ovarian fat pad weights reflect the final changes in body weight, although ADX saline animals at both day 7 and 51 had lower ovarian fat masses than sham-ADX D-Fen even though ADX saline body weight > sham-ADX D-Fen body weight. The anomaly in ovarian fat masses between adrenalectomised and sham-ADX-operated animals can possibly be explained by the lipid mobilisation effect of glucocorticoids. Glucocorticoids are known to promote the mobilisation of fat stores and cause specific relocation (as in Cushing's syndrome where fat distribution is seen to be localised

around shoulders and face). The reduction of ovarian fat in both adrenalectomised groups was seen at both days 7 and 51.

The trend for sham-ADX D-Fen animals to display higher corticosterone levels than sham-ADX saline animals (although non-significant) at both day 7 and 51 may be a reflection of the stimulation of the HPA axis by D-Fen. The stimulation of the HPA axis by D-Fen has been demonstrated by other investigators (Fuller *et al.* 1981, McElroy *et al.* 1984, Barbieri 1984, Holmes *et al.* 1982, De Souza *et al.* 1989, Serri and Rasio 1987). The levels of corticosterone, at the time of sacrifice, would be expected to be low, and together with the reduced levels of D-Fen and D-Nor-Fen at this time (18 hours following injection) make the interpretation of the results difficult. Initially, D-Fen would stimulate the HPA and produce elevated corticosterone levels compared to sham-ADX saline animals, but this effect would only be pronounced as long as the levels of D-Fen are maintained. So, although the results suggest a non-significant effect of D-Fen on corticosterone levels, at the time of sacrifice, the early response to D-Fen must be considered.

3.2. Effect of adrenalectomy in acute and chronic D-Fenfluramine treated female Wistar rats on the hypothalamic and hippocampal serotoninergic system.

3.2.1.

As 5-HT has been implicated in the control of the hypothalamic-pituitary-adrenal axis, and as fenfluramine is recognised to affect serotoninergic (5-HT) systems, the interactions of D-Fen and adrenalectomy on the serotoninergic system of the hypothalamus and hippocampus were investigated.

3.2.2. Effect of acute and chronic treatment on levels of hypothalamic 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), the 5-HT:5-HIAA ratios and tryptophan levels.

The hypothalamic levels of 5-HT (ng g^{-1} wet brain tissue), 5-HIAA (ng g^{-1} wet brain tissue), the ratio 5-HT:5-HIAA and levels of tryptophan (mg g^{-1} wet brain tissue) for the animals depicted in Figure 3.1. to 3.5. and Tables 3.1. and 3.2. are presented in Table 3.4. (see Chapter 2 for experimental protocols).

The 5-HT levels, at day 7, in sham-ADX D-Fen animals were significantly reduced to 50% control (ADX and sham-ADX saline levels) levels, ($p<0.01$), whilst ADX D-Fen animals demonstrated further reduced levels to approximately 20% control (ADX and sham-ADX saline levels), ($p<0.001$), which were significantly lower than sham-ADX D-Fen levels ($p<0.05$). There was no difference between ADX saline and sham-ADX saline groups. Two way ANOVA showed significant effects of surgery and D-Fen ($p=0.007$ and $p=0.021$ respectively). By day 51 5-HT levels of the ADX D-Fen animals were non-significantly different to sham-ADX D-Fen, but both were significantly reduced compared to saline treated animals, by two way ANOVA, ($p=0.003$).

5-HIAA levels were similarly reduced by 50% on day 7 in D-Fen treated animals compared to saline treated animals ($p=0.013$), the levels of 5-HIAA were lower in the

ADX D-Fen animals compared to sham-ADX D-Fen, but the effect was non-significant. No effect of surgery on 5-HIAA levels was seen. At day 51 no effect of surgery, by two way ANOVA, was seen whilst the effect of D-Fen treatment was significant ($p=0.009$). Sham-ADX D-Fen and ADX D-Fen levels of 5-HIAA were non-significantly different.

The ratio of 5-HT:5-HIAA is quoted as an indicator of possible changes in the turnover of 5-HT. Adrenalectomy versus sham-ADX surgery and D-Fen treatment versus saline both produce significant reductions in the ratio 5-HT:5-HIAA ($p<0.001$ and $p=0.004$ respectively). The joint effect of D-Fen and adrenalectomy potentiated the reduction in the ratio 5-HT:5-HIAA in ADX D-Fen animals (0.62 ± 0.15 - see table 3.4) compared to either sham-ADX D-Fen (1.45 ± 0.19 - see table 3.4) or ADX saline (1.44 ± 0.19 - see table 3.4), ($p<0.01$ in both cases). By day 51, there was no statistically significant difference in 5-HT:5-HIAA ratios between any of the groups.

No effect on hypothalamic tryptophan was seen with either D-Fen or adrenalectomy on day 7 or 51.

Table 3.4. The effects of acute and chronic D-Fen and adrenalectomy on the hypothalamic concentrations of 5-HT, 5-HIAA and tryptophan for the animal groups depicted in Figures 3.1. to 3.5 (Section 3.2).

Data points are means ± SEM. All data groups contain six animals.

See appendix 2 for ANOVA F values (reference number in parentheses).

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

Table 3.4.

	5-HT (ng g^{-1})		5-HIAA (ng g^{-1})		RATIO 5-HT:5-HIAA		TRYPTOPHAN (μg g^{-1})	
Treatment	Day 7	Day 51	Day 7	Day 51	Day 7	Day 51	Day 7	Day 51
Sham D-Fen	145 ± 36 **	592 ± 65 *	105 ± 23	269 ± 28 *	1.45 ± 0.19	2.22 ± 0.14	5.01 ± 0.39	5.57 ± 0.82 *
ADX D-Fen	54 ± 18 *** a	615 ± 58 *	82 ± 9 *	299 ± 40 *	0.62 ± 0.15 ** b	2.11 ± 0.21	5.06 ± 0.54	4.49 ± 0.53
Sham Saline	307 ± 21	939 ± 81	177 ± 15	411 ± 27	1.76 ± 0.06	2.28 ± 0.16	5.46 ± 0.53	3.85 ± 0.22
ADX Saline	331±127	939 ± 226	195 ± 57	434 ± 123	1.44 ± 0.19	2.22 ± 0.15	5.37 ± 0.67	4.80 ± 0.78

2 Way Anova

Sham v ADX	p=0.007 (1)	NS (4)	NS (7)	NS (10)	p=0.001 (13)	NS (16)	NS (19)	NS (22)
D-Fen v saline	p=0.021 (2)	p=0.003 (5)	p=0.013 (8)	p=0.009 (11)	p=0.004 (14)	NS (17)	NS (20)	NS (23)
Interaction	NS (3)	NS (6)	NS (9)	NS (12)	NS (15)	NS (18)	NS (21)	NS (24)

3.2.3. Effect of acute and chronic treatment on hippocampal 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), the ratio of 5-HT:5-HIAA and tryptophan levels.

The hippocampal levels of 5-HT (ng g^{-1} wet brain tissue), 5-HIAA (ng g^{-1} wet brain tissue), the ratio 5-HT:5-HIAA and levels of tryptophan (mg g^{-1} wet brain tissue) for the animals depicted in Figure 3.1. to 3.5. and Tables 3.1. and 3.2. are presented in Table 3.5

D-Fen significantly reduced 5-HT in the hippocampus compared to saline treatment ($p<0.001$). The 5-HT level in the sham-ADX D-Fen animals, at day 7, was reduced to 30% of the level seen in the saline treated group and ADX D-Fen 5-HT levels were further reduced to 10% of saline levels (although not significant different from sham-ADX D-Fen levels). By day 51 the reduction in 5-HT was reversed with 5-HT in the ADX D-Fen animals > sham-ADX D-Fen animal levels; both D-Fen animals having significantly reduced 5-HT levels compared to their saline controls ($p<0.001$ in both cases). No effect of adrenalectomy on hippocampal 5-HT surgery was seen.

5-HIAA levels were similarly reduced at day 7 with D-Fen ($p<0.001$) with sham-ADX D-Fen levels > ADX D-Fen levels (although non-significantly different). By day 51 a significant reduction in 5-HIAA levels was seen with D-Fen ($p=0.001$), with the levels in ADX D-Fen > sham-ADX D-Fen (again not significant). No effect of surgery was seen on day 7. Hippocampal 5-HIAA levels are significantly higher in adrenalectomised animals compared to their sham-ADX controls ($p=0.029$) at day 51.

The trend towards a reduction of the hippocampal ratio 5-HT:5-HIAA at day 7 was similar to that seen in the hypothalamus, although the effect of D-Fen versus saline was non-significant (with no difference between ADX saline and ADX D-Fen, and between sham-ADX saline and sham-ADX D-Fen). By day 51, D-Fen versus saline produced a significant reduction in the ratio 5-HT:5-HIAA ($p<0.001$), with no significant differences between the two D-Fen groups (although ADX D-Fen animals tended to have a lower ratio, as in the hypothalamus). No effect of surgery was seen.

Table 3.5. The effects of acute and chronic D-Fen and adrenalectomy on the hippocampal concentrations of 5-HT, 5-HIAA and tryptophan for the animal groups depicted in Figures 3.1. to 3.5 (Section 3.2).

Data points are means ± SEM. All data groups contain six animals.

See appendix 2 for ANOVA F values (reference number in parentheses).

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

Table 3.5.

	5-HT (ng g^{-1})		5-HIAA (ng g^{-1})		RATIO 5-HT:5-HIAA		TRYPTOPHAN (µg g^{-1})	
Treatment	Day 7	Day 51	Day 7	Day 51	Day 7	Day 51	Day 7	Day 51
Sham D-Fen	40 ± 16 **	122 ± 123 ***	45 ± 19 *	131 ± 21 ***	1.17 ± 0.28	0.92 ± 0.07 ***	5.48 ± 0.29	4.87 ± 0.81
ADX D-Fen	9 ± 4 **	276 ± 89 **	22 ± 10 **	240 ± 57 **	0.94 ± 0.29	1.07 ± 0.26 *	4.10 ± 0.38 b	5.56 ± 0.38
Sham Saline	154 ± 38	657 ± 117	111 ± 21	363 ± 41	1.31 ± 0.15	1.76 ± 0.10	5.12 ± 0.40	4.10 ± 0.30
ADX Saline	119 ± 33	742 ± 85	97±22	463 ± 56	1.14 ± 0.08	1.61 ± 0.03	4.92 ± 0.31	5.24 ± 0.31

2 Way Anova								
Sham v ADX	NS (1)	NS (4)	NS (7)	p=0.029 (10)	NS (13)	NS (16)	p=0.028 (19)	NS (22)
D-Fen v saline	p<0.001 (2)	p<0.001 (5)	p=0.001 (8)	p<0.001 (11)	NS (14)	p<0.001 (17)	NS (20)	NS (23)
Interaction	NS (3)	NS (6)	NS (9)	NS (12)	NS (15)	NS (18)	NS (21)	NS (24)

Adrenalectomy significantly reduced hippocampal tryptophan at day 7 (p=0.028). By day 51 both adrenalectomised groups had significantly elevated tryptophan compared to sham-ADX (p=0.016). No effect on hippocampal tryptophan was seen with D-Fen on either day 7 or 51.

3.2.4. Discussion.

Both hypothalamic and hippocampal 5-HT and 5-HIAA were reduced with chronic D-Fen treatment at day 7 and 51. This data supports the observations by other investigators (Kleven and Seiden 1989, Zaczek *et al.* 1990, Rowland 1986). D-Fen treatment similarly caused a reduction in the 5-HT:5-HIAA ratio at day 7 in both the hypothalamus and hippocampus, which has also been demonstrated by other groups (Fuller *et al.* 1978, Orosco *et al.* 1984, Rowland 1986). Chronic D-Fen administration for 51 days resulted in slight non-significant lowering of this ratio in the hypothalamus, with only the hippocampus maintaining a significant reduction in the ratio by day 51. With chronic D-Fen treatment, there was a recovery in the levels of 5-HT with hypothalamic 5-HT levels at day 7 and 51 being approximately 30% and 60% of saline controls respectively. Hippocampal 5-HT levels were, however, maintained at approximately 25% and 30% of saline controls at days 7 and 51 respectively.

The ratio of 5-HT:5-HIAA is quoted as an indicator of possible changes in the turnover of 5-HT. Adrenalectomy versus sham-ADX surgery and D-Fen treatment versus saline at day 7 both produce significant reductions in the ratio 5-HT:5-HIAA. The joint effect of D-Fen and adrenalectomy potentiated the reduction in the ratio 5-HT:5-HIAA in ADX D-Fen compared to either sham-ADX D-Fen or ADX saline. By day 51, there was no statistically significant difference in 5-HT:5-HIAA ratios between any of the groups. These results are discussed in depth in Chapter 4.

D-Fen had no effect on hippocampal tryptophan (also demonstrated [brain levels] by Costa *et al.* 1971). Adrenalectomy produced a decrease and increase in hippocampal tryptophan at days 7 and 51 respectively. In confirmation, Miller *et al.* 1978 demonstrated increases in brain tryptophan following long-term adrenalectomy. In contrast, neither D-Fen nor ADX had any effects on hypothalamic tryptophan.

3.3. Effect of adrenalectomy in acute and chronic D-Fenfluramine treated female Wistar rats on body composition and the metabolic fate of energy intake.

3.3.1.

The effect of D-Fen, adrenalectomy or of both treatments on the body composition of the animals of section 3.1. and 3.2. were assessed to quantify body weight changes to the specific alterations in body store of fat, protein and energy.

The total body protein, fat and energy content of the experimental animals described in sections 3.1. and 3.2. are presented in Table 3.6 (see chapter 2 for experimental protocols).

3.3.2. Effect of acute and chronic treatment on body protein.

Animals sacrificed on day 7 demonstrated significant effects of D-Fen versus saline and adrenalectomy versus sham-ADX in reducing body protein ($p=0.006$ and $p=0.018$ respectively). There were no significant reductions in protein in either ADX saline or sham-ADX D-Fen animals compared to sham-ADX saline animals, all having deposited approximately 10g in the study period of day 1 to 7 ($p<0.01$ compared to day 1 controls). ADX D-Fen animals, however, showed a significantly lower level of body protein compared to sham-ADX D-Fen, sham-ADX saline and ADX saline animals ($p<0.01$, $p<0.01$ and $p<0.05$ respectively), with a non-significant change compared to day 1 controls.

By day 51, no effect of D-Fen versus saline or adrenalectomy versus sham-ADX in reducing body protein was observed.

3.3.3. Effect of acute and chronic treatment on body lipid.

Unexpectedly, at day 7, fat deposition by D-Fen verses saline treatment had no significant effect, whilst adrenalectomy produced a significant reduction ($p=0.004$). The fat content of the animals was in the order sham-ADX saline > sham-ADX D-Fen > ADX D-Fen = ADX saline, with both ADX groups being significantly lower than the sham-ADX saline controls ($p<0.01$), with ADX D-Fen being non-significant from sham-ADX D-Fen. For the study period, days 1 to 7, both adrenalectomised groups showed no net change in fat deposition, with sham-ADX D-Fen and sham-ADX saline depositing approximately 2 and 4g respectively. By day 51, both ADX D-Fen and ADX saline had significantly reduced body fat compared to sham-ADX controls ($p<0.001$), with both adrenalectomy groups having similar values. Total body fat was in the order sham-ADX saline>sham-ADX D-Fen>ADX saline>ADX D-Fen, corresponding to fat deposition from day 1 to 51 of 27, 20, 10 and 7g respectively. No effect of D-Fen versus saline was seen, although the fat deposition in sham-ADX D-Fen animals was lowered compared to sham-ADX saline animals.

3.3.4. Effect of acute and chronic treatment on gross energy.

Total carcass energy, at day 7, showed a significant reduction in adrenalectomised rats ($p<0.001$), with no significant effect of D-Fen versus saline. Sham-ADX D-Fen animals were non-significantly different to sham-ADX saline animals. ADX saline animals had significantly reduced body energy content compared to sham-ADX saline ($p<0.05$), and ADX D-Fen animals having further reduced levels being significant from sham-ADX D-Fen and ADX saline animals ($p<0.001$ and $p<0.01$ respectively). The energy deposition was in the order sham-ADX saline = sham-ADX D-Fen > ADX saline > ADX D-Fen, which corresponds to a change of approximately +0.15MJ, 0MJ and -0.15MJ.

Table 3.6. The effects of acute and chronic D-Fen and adrenalectomy on total body protein (g animal^{-1}), fat (g animal^{-1}) and energy content (MJ animal^{-1}) for the animal groups depicted in Figures 3.1. to 3.5, and day 0 controls (Section 3.3).

Data points are means ± SEM. All data groups contain six animals.

See appendix 2 for ANOVA F values (reference number in parentheses).

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively) for respective sacrifice days.

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively) for respective sacrifice days.

Table 3.6.

	Protein (g animal^{-1})		Fat (g animal^{-1})		Energy (MJ animal^{-1})	
Treatment	Day 7	Day 51	Day 7	Day 51	Day 7	Day 51
Sham D-Fen	32.6 ± 1.6	48.3 ± 1.8	13.8 ± 0.6	31.7 ± 3.4	1.03 ± 0.05	1.79 ± 0.11
ADX D-Fen	26.6 ± 0.6 * b	51.3 ± 2.4	11.9 ± 0.9	18.7 ± 2.6 a	0.70 ± 0.04 ** c	1.41 ± 0.12
Sham Saline	34.3 ± 1.5	48.5 ± 2.1	15.8 ± 1.5	38.2 ± 2.7	1.01 ± 0.06	2.01 ± 0.13
ADX Saline	32.0 ± 1.5	50.6 ± 3.3	11.4±0.8 b	22.1 ± 3.3 c	0.85 ± 0.04 a	1.51 ± 0.14 b

2 Way Anova

Sham v ADX	p=0.006 (1)	NS (4)	p=0.004 (7)	p<0.001 (10)	p<0.001 (13)	p<0.001 (16)
D-Fen v saline	p=0.018(2)	NS (5)	NS (8)	NS (11)	NS (14)	NS (17)
Interaction	NS (3)	NS (6)	NS (9)	NS (12)	NS (15)	NS (18)

Day 1 control	24.1 ± 0.9	11.8 ± 0.8	0.85 ± 0.07

By day 51 the energy content of the animals similarly reflected the body weight profiles, with carcass content of sham-ADX saline > sham-ADX D-Fen > ADX saline > ADX D-Fen, corresponding to approximate increases from day 1 of +1.15MJ, +0.95MJ, +0.65MJ and +0.55MJ respectively. Day 51 animals showed significant reductions for adrenalectomy versus sham-ADX ($p<0.001$), with no significant effect of D-Fen versus saline.

3.3.5. Effect of acute and chronic treatment on the gross efficiency of food deposition.

One method for establishing the metabolic fate of food is to correlate the known amount of energy consumed during a study period (day 1 to either 7 or 51) to the changes in energy content. This factor is termed the gross energetic efficiency of food deposition (GEEFD). This can be illustrated by the case of an animal experiencing a change in energy composition of +100KJ associated with a gross energy intake of 1000KJ: the animal can be said to express a GEEFD of 10% (0.1). The GEEFD values can give an indication of changes in body weight of animals to discover if weight loss is solely a hypophagic factor, or whether a change in the usage of energy intake in addition has occurred. Reductions in GEEFD could be attributable to decreased efficiency of GI absorption, decreased efficiency of nutrient usage and/or an increase in the transfer of energy away from storage by the increase in energy expenditure of the animals (DIT, resting metabolic rate, thermogenesis).

The GEEFD for animals sacrificed on day 7 ($GEEFD_{day\ 7}$) and 51 ($GEEFD_{day\ 51}$) are presented in Figures 3.6A. and 3.6B. respectively. Adrenalectomy verses sham-ADX produces a significant reduction in $GEEFD_{day\ 7}$ and $GEEFD_{day\ 51}$ ($p<0.001$), with the effect of D-Fen verses saline being non-significant on both days. ADX saline $GEEFD_{day\ 7}$ is reduced compared to sham-ADX saline (although just non-significant), whilst ADX D-Fen is significantly reduced further, being significantly lower than sham-ADX saline, sham-ADX D-Fen and ADX saline animal groups ($p<0.001$, $p<0.001$ and $p<0.01$ respectively.

Figures 3.6.A and 3.6.B. Gross energetic efficiency of food deposition (GEEFD) for the animals shown in figures 3.1 to 3.5 and tables 3.1 to 3.6.

The following groups were studied; sham-ADX D-Fen, ADX D-Fen, sham-ADX saline and ADX saline for the acute (Figure 3.6.A) and chronic (Figure 3.6.B) study.

GEEFD was determined from the difference between the energy content of individual animals, calculated using the combustible energy values shown in table 3.6, on day 0 and termination, in relation to the energy consumption between day 0 and the sacrifice day. The GEEFD is expressed as the change in body energy content (KJ animal^{-1}) per unit energy consumption for period of study (KJ). (PRD chow energy content 12.02KJ g^{-1}).

Data points are means ± SEM. All data groups contain six animals.

2 way ANOVA shows significant effect of adrenalectomy versus sham-ADX surgery in animals sacrificed on day 7 and 51 with p values of <0.001 in both cases $F(1,24)=26.58$ and $F(1,24)=20.14$ respectively.

2 way ANOVA shows no significant effect of D-Fen versus saline in animals sacrificed on either day 7 or day 51.

Interaction between groups is seen on day 7 ($p=0.011$) $F(1,24)=7.95$.

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

Figure 3.6A

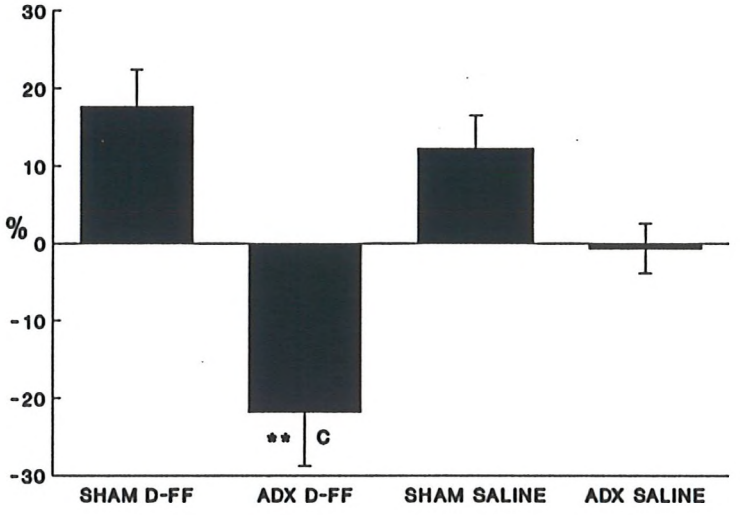
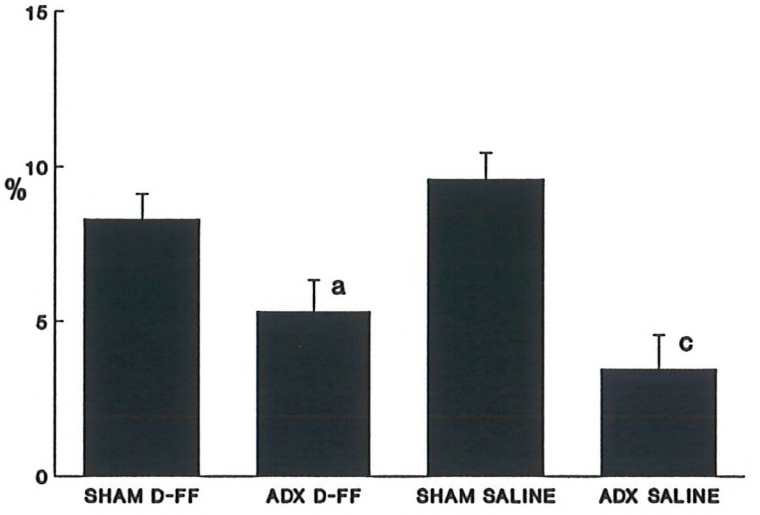


Figure 3.6B



$GEEFD_{day\ 7}$ values are in the order sham-ADX D-Fen = sham-ADX saline > ADX saline > ADX D-Fen, corresponding to $GEEFD_{day\ 7}$ values of approximately 15%, 0% and -20% respectively. Interaction between D-Fen and adrenalectomy, in reducing $GEEFD_{day\ 7}$, is seen ($p=0.011$).

The negative $GEEFD_{day\ 7}$ values of both adrenalectomised groups is postulated to be a result of the decreased usage of energy intake due to increases in the energy expenditure of the animals.

By day 51, the ADX D-Fen animals have restored GEEFD levels to the levels of ADX saline animals, which are both significantly lower than sham-ADX animals ($p<0.001$). $GEEFD_{day\ 51}$ values are in the order sham-ADX D-Fen = sham-ADX saline > ADX saline = ADX D-Fen, corresponding to $GEEFD_{day\ 51}$ values of approximately 8.5%, and 4% respectively.

3.3.6 Discussion.

The body composition data generally agrees with the changes in body weights observed, although there are some important points. Only the ADX D-Fen animals, at day 7, show a reduction in total body protein, which is a reflection of the lower body weight of the individuals, with both the sham-ADX D-Fen and ADX saline animals showing small reductions (non-significant) compared to sham-ADX saline animals (again a reflection in the reduced body weight, and the expected lower lean body mass). By day 51, all animals have similar body protein.

The total body lipid at both day 7 and 51 was reduced in adrenalectomised animals compared to sham-ADX animals. The large difference in ovarian fat mass in the ADX D-Fen animals compared to ADX saline animals is not reflected in the total lipid content of these animals, with ADX D-Fen = ADX saline < sham-ADX D-Fen < sham-ADX saline. Sham-ADX D-Fen shows slight reductions in total lipid compared to sham-ADX saline at both day 7 and 51 (as seen in the ovarian fat masses). The day 51 profiles of total lipid reflect the ovarian fat masses seen at this time in all treatment groups. The reduction

in total body lipid confirms the observations by other investigators of fenfluramine decreasing the deposition of fat in epidydymal pads (Brindley *et al.* 1985) and the reduction in body fat more markedly than protein and muscle in animals (Beyen *et al.* 1986, Fantino and Faion 1986).

Total energy of the animals reflects the changes in lipid and protein status, and the body weight profiles. The calorific value of fat is over ten times that of protein, meaning that changes in lipid mass will have a greater effect on the total energy composition of an individual than equivalent changes in protein mass. The large reduction in lipid observed in adrenalectomised animals is reflected in the lower energy composition of the animals. The ADX D-Fen animals have even further reduced total energy content compared to ADX saline by the lower levels of protein in comparison. D-Fen alone appears not to affect total body energy composition at day 7, but at day 51 the energy content is lower than the sham-ADX saline animals (although non-significant).

3.4. Effect of corticosterone-replacement on the response to adrenalectomy in acute D-Fenfluramine treated female Wistar rats.

3.4.1.

To establish whether the adrenal component in the potentiation of D-Fen anorexia by adrenalectomy is the removal of endogenous glucocorticoids, corticosterone was administered to adrenalectomised animals.

A similar protocol to section 3.1. (acute treatment) was adopted, except that the two adrenalectomy groups (ADX D-Fen and ADX saline) were duplicated and administered with corticosterone replacement (5mg kg^{-1}) in arachis oil carrier (10 mg ml^{-1}). All the other groups received arachis oil alone (0.5ml kg^{-1}). Arachis oil ± corticosterone was injected s.c. (intrascapular). D-Fen and saline administration, and surgery, was performed as previously reported in Section 3.1., with the initial corticosterone or vehicle administration at the time of surgery (1100-1300 hours) on day 3. Subsequent injections of arachis oil ± corticosterone was at the same time as D-Fen or saline injections (1700-1800 hours).

The animals were allowed to proceed as before, and were terminated on day 8.

3.4.2. Effect of corticosterone-replacement on acute treatment body weight profile.

The body weight profiles of the animal groups are presented in Figure 3.7. A significant effect of adrenalectomy verses sham-ADX was seen on days 3 to 8, and of D-Fen verses saline for days 2 to 8. The body weight profile of the ADX D-Fen, sham-ADX D-Fen, sham-ADX saline and ADX saline animal groups exhibited similar profiles as seen in Figure 3.1, in that D-Fen produces a reduction in body weight in both treated animals ($p<0.001$) and sham-ADX D-Fen animals body weight growth stabilised at a level below the sham-ADX saline animals. The ADX saline animals showed the reduction in body weight growth maintained below the sham-ADX saline

animals till the end of the study ($p<0.05$), with the ADX D-Fen animals maintaining body weights till the end of the study at a level of 20g, 25g and 30g below sham-ADX D-Fen, ADX saline and sham-ADX saline respectively ($p<0.001$ ADX D-Fen to all groups).

The two corticosterone-replacement groups, ADX D-Fen plus corticosterone (open double triangle) and ADX saline plus corticosterone (asterisk), are presented in Figure 3.7. The ADX saline plus corticosterone group followed a body weight profile identical to that of sham-ADX saline for the whole study, abolishing the effect of adrenalectomy. The ADX D-Fen plus corticosterone group do not completely mirror their supposed controls (sham-ADX D-Fen), but towards the end of the study the difference in body weights of these two groups decreases, so that from day 6 onwards there is no significant difference between them. The ADX D-Fen plus corticosterone group body weight is significantly higher than ADX D-Fen animals from day 5 onwards (day 5 $p<0.05$, day 6 $p<0.01$ and $p<0.001$ for days 7 and 8).

A significant effect of adrenalectomy verses sham-ADX and D-Fen verses saline was seen on days 3 ($p=0.040$), days 4 ($p=0.007$), days 5 to 8 ($p<0.001$) and days 2 to 8 ($p<0.001$) respectively.

Figure 3.7. The effect of corticosterone treatment on the changes in body weight of adrenalectomised rats treated with D-Fenfluramine.

The following groups were studied; sham-ADX saline (open square), ADX saline (open triangle), sham-ADX D-Fen (cross), ADX D-Fen (open circle), ADX D-Fen plus corticosterone treatment (open double triangle) and ADX saline plus corticosterone treatment (asterisk).

Data points are means ± SEM. All data groups contain five animals. The solid bar represents the period of daily D-Fen injections (10mg kg^{-1} day^{-1}) in sterile saline (10mg ml^{-1}) or sterile saline vehicle (1ml kg^{-1}) alone between 1700 and 1800 hours (i.p.).

2 way ANOVA shows significant effect of adrenalectomy versus sham-ADX surgery on days 3 to 8 with p values of $p=0.040$, $p=0.007$ and $p<0.001$ for days 5 to 8. See appendix 1 for F values.

2 way ANOVA shows significant effect of D-Fen versus saline on days 2 to 8 with a p value of <0.001 for all days. See appendix 1 for F values.

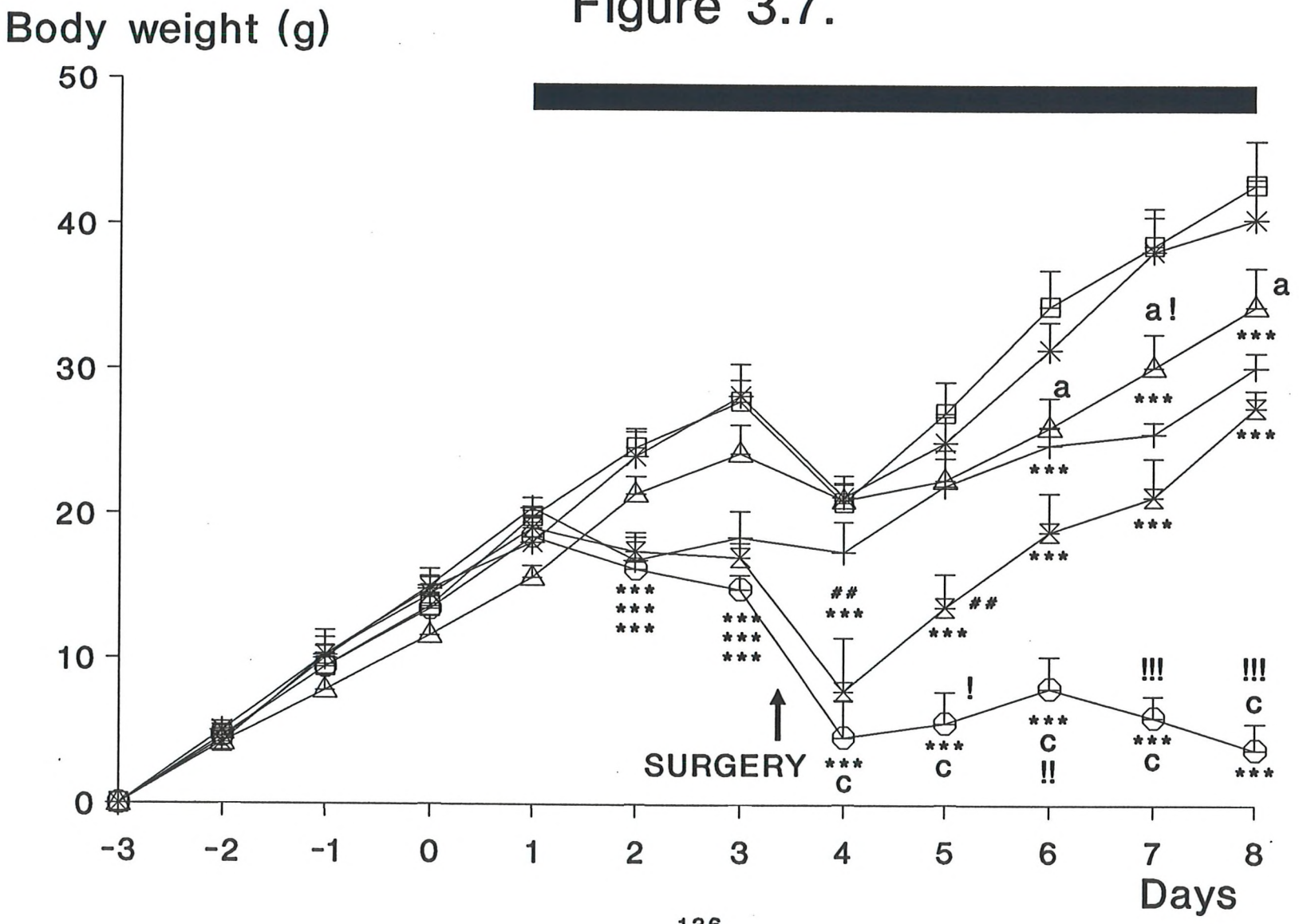
*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

!, !! and !!! indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone treatment groups and their group control (ADX saline and ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

#, ## and ### indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone treatment groups and their sham-ADX control group (sham-ADX saline and sham-ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

Figure 3.7.


Body weight (g)
50
40
30
20
10
0
-3
-2
-1
0
1
2
3
4
5
6
7
8
Days
SURGERY
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c
c
c
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3.4.3. Effect of corticosterone-replacement on acute food intake profile.

The food intake profiles of these animals are presented in Figure 3.8. Food intake, for the four appropriate groups, mirror the profile seen in the animals for section 3.1 (Figure 3.2), with a significant effect of adrenalectomy verses sham-ADX and D-Fen verses saline on days 3 to 7 and 1 to 7 respectively (see legend for p values).

The food intake profiles for the corticosterone-replacement animals, following surgery, did not exactly mirror their sham-ADX equivalents. The ADX D-Fen plus corticosterone animals show food intake levels 200% of sham-ADX D-Fen levels, whilst the ADX saline plus corticosterone levels are 30% of sham-ADX saline levels on day 3. However, by day 5 there was no difference in food intake levels between sham-ADX D-Fen and ADX D-Fen + corticosterone nor between sham-ADX saline and ADX saline + corticosterone. ADX D-Fen plus corticosterone animals have significantly elevated food intakes compared to ADX D-Fen from day 3 onwards ($p<0.001$), with ADX saline plus corticosterone significantly higher than ADX saline on days 4, 6 and 7 only ($p<0.001$, $p<0.001$ and $p<0.05$ respectively).

Figure 3.8. The effect of corticosterone treatment on the daily food intake of adrenalectomised rats treated with D-Fenfluramine.

The following groups were studied; sham-ADX saline (open square), ADX saline (open triangle), sham-ADX D-Fen (cross), ADX D-Fen (open circle), ADX D-Fen plus corticosterone treatment (open double triangle) and ADX saline plus corticosterone treatment (asterisk).

Data points are means ± SEM. All data groups contain five animals. The solid bar represents the period of daily D-Fen injections (10mg kg^{-1} day^{-1}) in sterile saline (10mg ml^{-1}) or sterile saline vehicle (1ml kg^{-1}) alone between 1700 and 1800 hours (i.p.).

2 way ANOVA shows significant effect of adrenalectomy versus sham-ADX surgery on days 3 to 7 with p values of $p=0.048$ for day 4 and <0.001 for rest. See appendix 1 for F values.

2 way ANOVA shows significant effect of D-Fen versus saline on days 1 to 7 with a p value of <0.001 for days 1 to 3, days 4 and 5 $p=0.001$, day 6 $p=0.008$ and day 7 $p=0.006$. See appendix 1 for F values.

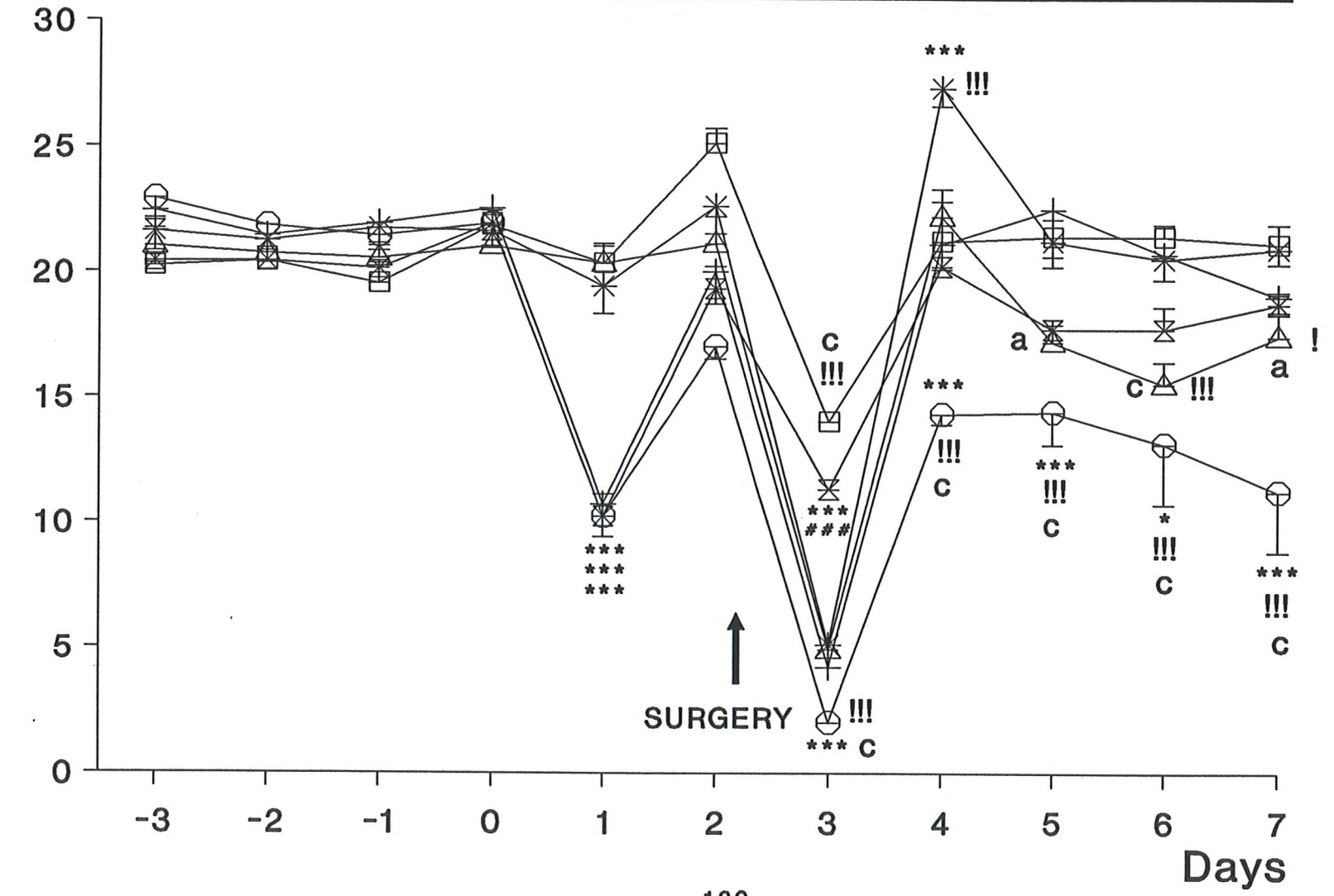
*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

!, !! and !!! indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$respectively) by Student Newman-Keul's procedure between corticosterone replacement groups and their group control (ADX saline and ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

#, ## and ### indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone replacement groups and their sham-ADX control group (sham-ADX saline and sham-ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

Figure 3.8.


Daily food intake (g)
30
25
20
15
10
5
0
-3
-2
-1
0
1
2
3
4
5
6
7
Days
SURGERY
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3.4.4. Effect of corticosterone-replacement on serum glucose and insulin, and liver and ovarian fat pad mass.

Serum glucose and insulin, liver and ovarian fat pad weights for the above animals are presented in Table 3.7. Sham-ADX D-Fen, ADX D-Fen, sham-ADX saline and ADX saline all showed similar profiles to that exhibited in Table 3.1. Corticosterone replacement reversed decreases in serum glucose and partially restored insulin levels towards their sham-ADX controls. ADX D-Fen plus corticosterone and ADX saline plus corticosterone liver and ovarian fat pad weights were fully restored to their respective sham-ADX controls, with both ADX D-Fen plus corticosterone and ADX saline plus corticosterone having significantly increased liver and ovarian fat weights compared to ADX D-Fen and ADX saline respectively (see table for p values).

3.4.5. Effect of corticosterone-replacement on total food intake and change in body weight.

The total food intake and the change in body weight for the period day 1 to 8 are presented in Figure 3.8. Sham-ADX D-Fen, ADX D-Fen, sham-ADX saline and ADX saline all showed similar profiles to that of the animal groups in Table 3.2. Corticosterone replacement restored the food intake of both groups to their sham-ADX controls. ADX D-Fen plus corticosterone and ADX saline plus corticosterone (together with sham-ADX D-Fen and sham-ADX saline) show significantly elevated food intakes compared to ADX D-Fen and ADX saline respectively ($p<0.001$ in all cases). Body weight profiles were similarly restored with ADX D-Fen plus corticosterone and ADX saline plus corticosterone significantly higher than ADX D-Fen and ADX saline ($p<0.001$ and $p<0.05$ respectively).

Table 3.7. The effect of corticosterone treatment to adrenalectomised rats receiving D-Fen on serum glucose (mM), insulin ($\mu U\ ml^{-1}$), liver weights (g) and ovarian fat mass weights (g) for the animal groups depicted in Figures 3.7. and 3.8 (Section 3.4).

Data points are means ± SEM. All data groups contain five animals.

See appendix 2 for ANOVA F values (reference number in parentheses).

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

!, !! and !!! indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone replacement groups and their group control (ADX saline and ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

#, ## and ### indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone replacement groups and their sham-ADX control group (sham-ADX saline and sham-ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

Table 3.7.

Treatment	Serum Glucose (mM)	Serum Insulin ($\mu U\ ml^{-1}$)	Liver weight (g)	Ovarian Fat Pad Weight (g)
Sham D-Fen	5.82 ± 0.11	74.0 ± 16.4	7.49 ± 0.23	0.15 ± 0.01 *
ADX D-Fen	5.33 ± 0.13 !! b	48.4 ± 12.6	5.35 ± 0.58 *** !!! c	0.06 ± 0.01 *** !! a
Sham Saline	5.68 ± 0.06	64.0 ± 17.3	8.28 ± 0.32	0.25 ± 0.04
ADX Saline	5.48 ± 0.07	45.4 ± 18.2	6.99 ± 0.39 b	0.10 ± 0.01 !
ADX D-Fen + Corticosterone	5.68 ± 0.16	59.2 ± 1.3	7.60 ± 0.14	0.16 ± 0.01
ADX Saline + Corticosterone	5.62 ± 0.13	54.0 ± 11.0	8.06 ± 0.41	0.18 ± 0.04

2 Way Anova				
Sham v ADX	p=0.002 (1)	NS (4)	p<0.001 (7)	p<0.001 (10)
D-Fen v saline	NS (2)	NS (5)	p=0.008 (8)	p=0.009 (11)
Interaction	NS (3)	NS (6)	NS (9)	NS (12)

Table 3.8. The effect of corticosterone treatment to adrenalectomised rats receiving D-Fen on total food intake and the change in body weight for the period from the initiation of D-Fen treatment (day 1) until termination (on day 8) for the animal groups depicted in Figures 3.7. and 3.8 (Section 3.4).

Data points are means ± SEM. All data groups contain five animals.

See appendix 2 for ANOVA F values (reference number in parentheses).

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

!, !! and !!! indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone replacement groups and their group control (ADX saline and ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

#, ## and ### indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone replacement groups and their sham-ADX control group (sham-ADX saline and sham-ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

Table 3.8.

Treatment	Food Intake	Change in BW
Sham D-Fen	114.8 ± 0.6 ***	10 ± 1 ***
ADX D-Fen	82.2 ± 2.9 *** c	-13 ± 2 *** c
Sham Saline	144.5 ± 3.5	23 ± 2
ADX Saline	118.7 ± 3.5 c	17 ± 2 a
ADX D-Fen + Corticosterone	118.4 ± 2.3 !!!	8 ± 1 !!!
ADX Saline + Corticosterone	131.7 ± 2.4 !!! ##	22 ± 1 !

2 Way Anova		
Sham v ADX	$p<0.001$ (1)	$p<0.001$ (4)
D-Fen v saline	$p<0.001$ (2)	$p<0.001$ (5)
Interaction	$p=0.018$ (3)	$p<0.001$ (6)

3.4.6. Effect of corticosterone-replacement on hypothalamic 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), the ratio 5-HT:5-HIAA and tryptophan levels.

The hypothalamic profile of 5-HT (ng g^{-1} wet brain tissue), 5-HIAA (ng g^{-1} wet brain tissue), the ratio 5-HT:5-HIAA and levels of tryptophan (mg g^{-1} wet brain tissue) for the corticosterone replacement animals are presented in Table 3.9. (see Chapter 2 for experimental protocols).

The hypothalamic profile of 5-HT, 5-HIAA, the ratio 5-HT:5-HIAA and tryptophan in sham-ADX D-Fen, ADX D-Fen, sham-ADX saline and ADX saline animals displayed a similar trend to that observed in section 3.1. The levels of 5-HT and 5-HIAA in the ADX D-Fen plus corticosterone animals were non-significantly different from the levels observed in ADX D-Fen animals, and the ratio 5-HT:5-HIAA (1.7) was only partially restored to sham-ADX D-Fen levels (2.2), although it remained non-significantly different, with the ADX D-Fen plus corticosterone 5-HT:5-HIAA ratio being significantly higher than the ADX D-Fen 5-HT:5-HIAA ratio (0.90), ($p<0.01$). The levels of both 5-HT and 5-HIAA in the ADX saline plus corticosterone animals are significantly lower than sham-ADX saline levels ($p<0.01$ in both cases), although the ratio 5-HT:5-HIAA is non-significantly different from the sham-ADX saline level.

No effect on hypothalamic tryptophan of surgery, D-Fen or corticosterone replacement was seen.

Table 3.9. The effects of corticosterone treatment to adrenalectomised rats receiving D-Fen on the hypothalamic concentrations of 5-HT (ng g^{-1}), 5-HIAA (ng g^{-1}) and tryptophan (μg g^{-1}) for the animal groups depicted in Figures 3.7. and 3.8 (section 3.4).

Data points are means ± SEM. All data groups contain five animals.

See appendix 2 for ANOVA F values (reference number in parentheses).

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

!, !! and !!! indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone replacement groups and their group control (ADX saline and ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

#, ## and ### indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone replacement groups and their sham-ADX control group (sham-ADX saline and sham-ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

Table 3.9.

Treatment	5-HT (ng g^{-1})	5-HIAA (ng g^{-1})	RATIO 5-HT:5-HIAA	TRYPTOPHAN (µg g^{-1})
Sham D-Fen	680 ± 51 ***	311 ± 17 ***	2.22 ± 0.25 *	9.39 ± 0.91
ADX D-Fen	348 ± 74 ***	297 ± 29 ***	0.94 ± 0.29 *** !! a	8.30 ± 1.25
Sham Saline	1845 ± 226	568 ± 54	3.16 ± 0.37	7.47 ± 0.46
ADX Saline	1500 ± 208	561 ± 42	2.63 ± 0.20	11.30 ± 2.45
ADX D-Fen + Corticosterone	410 ± 57	269 ± 37	1.67 ± 0.33	10.81 ± 1.61
ADX Saline + Corticosterone	1132 ± 120 ##	382 ± 45 !! ##	2.90 ± 0.13	11.63 ± 0.40

2 Way Anova

Sham v ADX	NS (1)	NS (4)	p=0.027 (7)	NS (10)
D-Fen v saline	P<0.001 (2)	p<0.001 (5)	P=0.001 (8)	NS (11)
Interaction	NS (3)	NS (6)	NS (9)	NS (12)

3.4.7. Effect of corticosterone-replacement on hippocampal 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), the ratio 5-HT:5-HIAA and tryptophan levels.

The hippocampal profile of 5-HT (ng g^{-1} wet brain tissue), 5-HIAA (ng g^{-1} wet brain tissue), the ratio 5-HT:5-HIAA and levels of tryptophan (mg g^{-1} wet brain tissue) for the corticosterone replacement animals are presented in Table 3.10. (see Chapter 2 for experimental protocols).

The hippocampal profile of 5-HT, 5-HIAA, the ratio 5-HT:5-HIAA and tryptophan in sham-ADX D-Fen, ADX D-Fen, sham-ADX saline and ADX saline animals displayed a similar trend to that observed in section 3.1, except that ADX D-Fen did not display the lowering of 5-HT and 5-HIAA compared to sham-ADX D-Fen, which is due to the large variation seen in the 5-HT levels in these animals, which presented a 5-HT:5-HIAA ratio 2 fold higher than sham-ADX D-Fen.

Corticosterone replacement animals had similar 5-HT, 5-HIAA and ratio levels compared to their sham-ADX controls, except that 5-HIAA in ADX saline plus corticosterone was significant lower compared to sham-ADX saline ($p<0.05$).

No effect of surgery, D-Fen or corticosterone replacement on hippocampal tryptophan was seen.

Table 3.10. The effects of corticosterone treatment to adrenalectomised rats receiving D-Fen on the hippocampal concentrations of 5-HT (ng g^{-1}), 5-HIAA (ng g^{-1}) and tryptophan (μg g^{-1}) for the animal groups depicted in Figures 3.7. and 3.8 (section 3.4).

Data points are means ± SEM. All data groups contain five animals.

See appendix 2 for ANOVA F values (reference number in parentheses).

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective surgical saline control groups (sham-ADX saline and ADX saline to sham-ADX D-Fen and ADX D-Fen respectively).

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between respective sham-ADX and ADX drug groups (sham-ADX saline and sham-ADX D-Fen to ADX saline and ADX D-Fen respectively).

!, !! and !!! indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone replacement groups and their group control (ADX saline and ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

#, ## and ### indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between corticosterone replacement groups and their sham-ADX control group (sham-ADX saline and sham-ADX D-Fen to ADX saline plus corticosterone and ADX D-Fen plus corticosterone respectively).

Table 3.10.

Treatment	5-HT (ng g^{-1})	5-HIAA (ng g^{-1})	RATIO 5-HT:5-HIAA	TRYPTOPHAN (µg g^{-1})
Sham D-Fen	203 ± 29 ***	151 ± 16 ***	1.36 ± 0.16	8.15 ± 1.80
ADX D-Fen	463 ± 71 ** a	145 ± 14 ***	3.38 ± 0.68 *** ! b	11.64 ± 2.38
Sham Saline	952 ± 120	458 ± 48	2.08 ± 0.20	8.09 ± 1.33
ADX Saline	826 ± 103	482 ± 37	1.70 ± 0.14	9.88 ± 2.27
ADX D-Fen + Corticosterone	323 ± 37	203 ± 61	2.02 ± 0.40	10.84 ± 1.82
ADX Saline + Corticosterone	735 ± 48	304 ± 32 #	2.49 ± 0.18	11.88 ± 1.53

2 Way Anova

Sham v ADX	NS (1)	NS (4)	NS (7)	NS (10)
D-Fen v saline	p<0.001 (2)	p<0.001 (5)	NS (8)	NS (11)
Interaction	P=0.048 (3)	NS (6)	NS (9)	P=0.025 (12)

3.4.8. Serum corticosterone levels.

Serum corticosterone levels in the two adrenalectomised (with arachis oil control) animal groups (ADX D-Fen and ADX saline) were below the limits of quantification.

The corticosterone values for the sham-ADX D-Fen and sham-ADX saline (with arachis oil control) animals were 255±103 and 341±106 ng ml^{-1} respectively, whilst the ADX D-Fen plus corticosterone and ADX saline plus corticosterone serum corticosterone levels were 122±37 and 191±75ng ml^{-1}. There was no significant difference between sham-ADX D-Fen and sham-ADX saline, and between ADX D-Fen plus corticosterone and ADX saline plus corticosterone. The only significant difference occurred between sham-ADX saline and ADX D-Fen plus corticosterone ($p<0.05$). Both corticosterone replacement animals displayed reduced serum levels compared to sham-ADX animals (sham-ADX saline and D-Fen groups verses ADX saline and D-Fen corticosterone replacement groups by 2 way ANOVA $p<0.01$). The lower corticosterone levels could possibly of been a reflection of the exhaustion of corticosterone in the arachis oil pool, as the animals were sacrificed between 18 to 20 hours following the last corticosterone injection.

3.4.9. Discussion.

Corticosterone replacement would appear to reverse the effects of adrenalectomy in both the saline and D-Fen treated adrenalectomised animals.

The final body weights (day 8) of ADX D-Fen plus corticosterone and ADX saline plus corticosterone are restored to their respective sham-ADX animal controls (sham-ADX D-Fen and sham-ADX saline respectively). The profile of ADX saline plus corticosterone mirrors that of the sham-ADX saline animals, but the profile of the ADX D-Fen plus corticosterone animals is off-set by a fall in body weight following surgery which is greater that that seen with the sham-ADX D-Fen animals, and takes until the end of the study to be restored to the sham-ADX D-Fen level. This observation is further explained in Chapter 4.

Corticosterone replacement restored serum glucose and insulin to their respective sham-ADX controls, and restored fully the reductions in liver and ovarian fat masses associated in the two adrenalectomy groups. The only discrepancy in the total food intake occurs in the ADX saline plus corticosterone animals. This group has a significantly lower total food intake compared to the sham-ADX saline group and may be a reflection of the lower pre-surgical levels in the corticosterone replacement animals and/or the failure to reproduce sham-ADX-operated conditions immediately following surgery in terms of blood levels of corticosterone.

The decrease in hypothalamic 5-HT system associated with adrenalectomy in D-Fen treated animals is partially restored to the sham-ADX D-Fen profile by the replacement of corticosterone, together with an increase in the 5-HT:5-HIAA ratio in the ADX D-Fen plus corticosterone animals compared to ADX D-Fen to the level seen in sham-ADX D-Fen animals. This suggests that the adrenal factor removed responsible for the reduction of hypothalamic 5-HT and the reduction in the ratio of 5-HT:5-HIAA ratio observed in ADX D-Fen animals compared to sham-ADX D-Fen is corticosterone. Less information can be obtained from the hippocampal data because of the large variation associated with 5-HT, and the large 5-HT:5-HIAA ratio. Although, adrenalectomy can be seen to restore the 5-HT:5-HIAA ratio in both corticosterone replacement groups to their respective sham-ADX controls. The significance of these observations is discussed further in Chapter 4.

3.5. Effect of adrenalectomy in acute D-Fenfluramine treated female Wistar rats on hypothalamic corticotrophin-releasing factor (CRF).

3.5.1

The stimulation of the hypothalamic-pituitary-adrenal (HPA) axis by 5-HT, and D-Fen, is well documented (Fuller and Snoddy 1980, McElroy *et al.* 1984, Van de Kar *et al.* 1983). It is hypothesised that one or more of the components of the HPA axis plays a role in the body weight-reducing effects of D-Fen. Corticotrophin-releasing factor, in particular, plays a fundamental role.

Sections 3.2 and 3.4. demonstrated that ADX D-Fen animals have increased hypothalamic 5-HT activity. A possible mechanism for the potentiation of D-Fen anorexia by adrenalectomy is postulated to be a result of increased activity of hypothalamic activity in these animals. Hypothalamic CRF content is raised in adrenalectomised animals (see Chapter 1.), and 5-HT/D-Fen both stimulate the release of CRF. The rise in CRF is postulated to be one of the factors contributing to the anorexia associated with adrenalectomy. The increase in hypothalamic CRF dynamics, associated with adrenalectomy, may, with D-Fen treatment, cause a stimulation of the release of the high levels of CRF associated with adrenalectomy to further inhibit food intake and increase SNS activity, compared to either adrenalectomy or D-Fen treatment alone.

3.5.2. Immunocytochemical detection of CRF.

Initial studies began with immunocytochemical (ICC) localisation of CRF containing regions within the CNS (see Chapter 2.7 for protocol). Initially, ICC failed to detect CRF except within the end terminals of the medien eminence (ME). CRF is, however, located within many brain regions (seen Chapter 1.1.2.2.). To detect CRF within these other brain regions it was deemed necessary to administer colchicine (i.c.v) to block CRF transport from the cell bodies by axonal transport to the end terminals. Colchicine

(and other drugs including vinca alkaloids, podophyllotoxin and griseofulvin) bind to microtubules and cause them to disrupt into subunits. Microtubules play an essential in maintenance of intracellular structure, the movement and exocytotic release of secretory products. Colchicine (75μg $20ml^{-1}$) is injected into the lateral ventricle at the same time as adrenalectomy or sham-ADX surgery under pentobarbitone anaesthesia (see Chapter 2 for experimental protocol) on day 3. The animals were sacrificed 48 hours later on day 5. Colchicine completely abolished food intake in all rats, and of the 24 rats originally assigned to receive colchicine, only 5 survived to day 5. Colchicine treatment revealed positive CRF staining in the PVN region of the hypothalamus. Because the process of the potentiation of D-Fen anorexia by adrenalectomy is a dynamic process involving the modulation of food intake, use of ICC as an investigative technique was deemed inappropriate for this study and not pursued further.

3.5.3. IRMA of whole hypothalamic CRF.

The direct measurement of hypothalamic CRF of tissue by IRMA (see Chapter 2.8 for protocol), in hindsight, would allow changes in hypothalamic CRF to be measured quantitatively, whilst ICC detection of CRF would only establish the total number of CRF-positive cells.

Animals for IRMA measurement of CRF followed the same protocol as animals presented in section 3.1., to produce 4 groups:

- sham-ADX D-Fen
- ADX D-Fen
- sham-ADX saline
- ADX saline

See Chapter 2. for experimental protocol and tissue dissection.

The animals were sacrificed on day 6. Figure 3.9. shows the hypothalamic (including ME and pituitary stalk) level of CRF expressed as ng g^{-1} wet tissue.

Sham-ADX saline animals show hypothalamic levels of CRF of 22.2 ± 1.9 ng g^{-1} (range 14.4-28.0) with ADX saline CRF levels of 31.7 ± 1.8 ng g^{-1} (range 26.2-37.1), which were significantly different from each other ($p<0.05$).

Sham-ADX D-Fen animals level of CRF were 19.1 ± 3.2 ng g^{-1} (range 5.1-30.3) with ADX D-Fen CRF levels of 9.1 ± 1.7 ng g^{-1} (range 2.5-19.7). ADX D-Fen CRF levels were significantly lower than sham-ADX D-Fen, sham-ADX saline and ADX saline CRF levels ($p<0.01$, $p<0.001$ and $p<0.001$ respectively).

3.5.4. Discussion.

This increase of hypothalamic CRF, in the adrenalectomised saline-treated animals, is postulated to be a result of the loss of negative feedback inhibition by corticosterone and confirms the observations of other investigators (Eberwine and Roberts 1984, Kovács *et al.* 1986, Sawchenko 1987, Kovács and Makara 1988, Vale *et al.* 1983, Beyer *et al.* 1988).

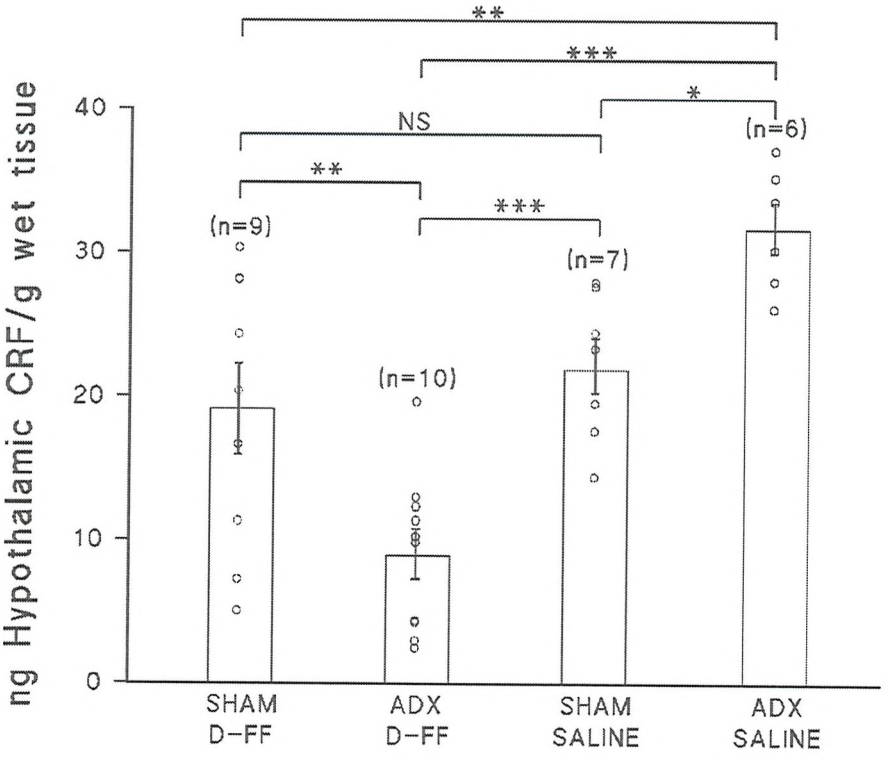
The reduction in the hypothalamic levels of CRF, by D-Fen, in ADX D-Fen animals, is taken as an index of stimulation of CRF release from CRF-containing cells, by the depletion of storage without replenishment. Other investigators have demonstrated that D-Fen (and 5-HT) causes a release of CRF from the hypothalamus (Calogero *et al.* 1989, Jones *et al.* 1976, Gibbs and Vale 1983, Nakagami *et al.* 1986, Jones and Hillhouse 1977, Holmes *et al.* 1982) and thus confirms this observation. The reduced hypothalamic CRF in ADX D-Fen animals compared to either sham-ADX D-Fen or ADX saline animals may be due to the increased hypothalamic 5-HT activity associated with these animals (see section 3.2.), causing an increase in CRF release compared to the sham-ADX D-Fen animals.

Figure 3.9. The effect of acute D-Fen and adrenalectomy on hypothalamic corticotropin-releasing factor (ng g^{-1} wet tissue)

See Section 2.5 for experimental protocols.

Data is shown as mean ± SEM with plots of individual data points (open circles).

Analysis by Student Newman-Keul's procedure between groups is shown by *, ** and *** ($p<0.05$, $p<0.01$ and $p<0.001$ respectively), together with non-significant comparisons.



3.6 Effect of adrenalectomy in acute and chronic D-Fenfluramine treatment, and of corticosterone-replacement in acute D-Fenfluramine treated adrenalectomised female Wistar rats on the brain and serum levels of D-Fen and its primary metabolite, D-Nor-Fen.

3.6.1.

A possible mechanism for the potentiation of D-Fen's action by the removal of endogenous glucocorticoids could involve changes in the metabolism of D-Fen into D-Nor-Fen and/or the modification of the pharmacokinetic profile of one or both of these compounds. The effect of glucocorticoids on the modulation of D-Fen and D-Nor-Fen metabolism and excretion is unknown.

3.6.2. Effect of adrenalectomy in acute and chronic D-Fen treated female Wistar rats on brain and serum levels of D-Nor-Fen, D-Fen and the ratio D-Nor-Fen:D-Fen.

Brain and serum levels of D-Nor-Fen, D-Fen and the ratio D-Nor-Fen:D-Fen for animals sacrificed on day 7 and 51 (section 3.1., 3.2. and 3.3.) are presented in Table 3.11.

ADX D-Fen animals sacrificed on day 7 showed significantly elevated brain D-Nor-Fen and D-Fen levels of 2.6 and 5.5 fold ($p<0.001$ in both cases) compared to sham-ADX D-Fen animals, with the ratio of D-Nor-Fen:D-Fen in the ADX D-Fen significantly lowered to 50% of sham-ADX D-Fen levels ($p<0.05$). Serum levels of D-Nor-Fen and D-Fen were similar to brain levels, with a significant 1.7 and 2.9 fold increases respectively of D-Nor-Fen and D-Fen ($p<0.01$ and $p<0.05$ respectively) in ADX D-Fen animals compared to sham-ADX D-Fen animals, and a significant reduction of the D-Nor-Fen:D-Fen ratio to 70% of sham-ADX D-Fen levels ($p<0.05$).

Table 3.11. The effect of acute and chronic D-Fen and adrenalectomy on brain and serum levels of D-Nor-Fen and D-Fen for the animal groups depicted in Figures 3.1. to 3.5 (Section 3.1).

Data points are means ± SEM. Data groups contain six animals.

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between sham-ADX D-Fen and ADX D-Fen animals for respective sacrifice days.

Table 3.11.

Treatment	BRAIN			SERUM		
	D-Nor-Fen ($\mu g\ g^{-1}$)	D-Fen ($\mu g\ g^{-1}$)	RATIO D-Nor-Fen:D-Fen	D-Nor-Fen ($ng\ ml^{-1}$)	D-Fen ($ng\ ml^{-1}$)	RATIO D-Nor-Fen:D-Fen
Sham D-Fen (Day 7)	5.02 ± 0.51	1.77 ± 0.26	3.08 ± 0.37	114 ± 12	47 ± 6	2.50 ± 0.28
ADX D-Fen (Day 7)	13.18 ± 1.11 ***	9.48 ± 2.03 ***	1.62 ± 0.29 *	192 ± 22 **	136 ± 40 *	1.72 ± 0.30 *
Sham D-Fen (Day 51)	11.32 ± 0.88	9.60 ± 1.51	1.31 ± 0.20	189 ± 14	145 ± 19	1.32 ± 0.10
ADX D-Fen (Day 51)	11.45 ± 1.15	6.99 ± 0.99	1.68 ± 0.12	194 ± 25	112 ± 18	1.77 ± 0.10

By day 51, ADX D-Fen levels of both brain and serum D-Nor-Fen and D-Fen were not significantly different from sham-ADX D-Fen animals. The ratio of D-Nor-Fen:D-Fen was similarly not significantly different between the two groups. Therefore the increased D-Fen and D-Nor-Fen and reduced D-Nor-Fen:D-Fen ratio noted in the ADX D-Fen animals compared to the sham-ADX D-Fen animals is no longer evident at day 51.

3.6.3 Effect of corticosterone-replacement and adrenalectomy in D-Fen treated female Wistar rats on brain and serum levels of D-Nor-Fen, D-Fen and the ratio D-Nor-Fen:D-Fen.

The brain and serum levels of D-Nor-Fen and D-Fen, with the ratio D-Nor-Fen:D-Fen, in the corticosterone replacement animals seen in section 3.4. are presented in Table 3.12.

A similar pattern to the day 7 animals (section 3.1 to 3.3) was seen in brain D-Nor-Fen and D-Fen, with statistically significant 2.2 and 6.7 fold increases respectively in levels in the ADX D-Fen animals compared to sham-ADX D-Fen animals ($p<0.001$ and $p<0.01$ respectively). The ratio D-Nor-Fen:D-Fen was also similarly significantly 40% lower in the ADX D-Fen animals compared to sham-ADX D-Fen animals ($p<0.001$). Serum levels of D-Nor-Fen and D-Fen were also similarly significantly elevated by 1.5 and 4.7 fold respectively in ADX D-Fen animals compared to sham-ADX D-Fen ($p<0.05$ and $p<0.01$ respectively). The ratio D-Nor-Fen:D-Fen was similarly significantly 36% of the sham-ADX D-Fen level ($p<0.001$).

Corticosterone replacement has some interesting results. Brain D-Nor-Fen and D-Fen were significantly lower in the ADX D-Fen plus corticosterone animals compared to ADX D-Fen animals ($p<0.001$ in both cases), and non-significantly different from sham-ADX D-Fen levels (although D-Fen was 50% sham-ADX D-Fen levels). The ratio D-Nor-Fen:D-Fen was significantly 1.9 and 5.1 fold higher in the ADX D-Fen plus corticosterone animals compared to sham-ADX D-Fen and ADX D-Fen respectively ($p<0.001$ in both cases). One animal in the ADX D-Fen plus corticosterone displayed non-detectable levels of D-Fen, and was not included in the D-Fen table and subsequent D-Nor-Fen:D-Fen analysis.

Table 3.12. The effect of corticosterone treatment on the effect of acute and chronic D-Fen and adrenalectomy on brain and serum levels of D-Nor-Fen and D-Fen for the animal groups depicted in Figures 3.7. and 3.8 (Section 3.4).

Data points are means ± SEM. All data groups contain five animals.

BLQ indicates that all samples were below the limits of quantification.

In the group receiving ADX D-Fen plus corticosterone, one animal showed no detectable level of brain D-Fen and is not included in the table, and in the subsequent calculation of the ratio D-Nor-Fen to D-Fen.

*, ** and *** indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between sham-ADX D-Fen and ADX D-Fen animals.

a, b and c indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between ADX D-Fen and ADX D-Fen + corticosterone replacement.

!, !! and !!! indicate a significant difference ($p<0.05$, $p<0.01$ and $p<0.001$ respectively) by Student Newman-Keul's procedure between sham-ADX D-Fen and ADX D-Fen + corticosterone replacement.

Table 3.12.

Treatment	BRAIN			SERUM		
	D-Nor-Fen ($\mu g\ g^{-1}$)	D-Fen ($\mu g\ g^{-1}$)	RATIO D-Nor-Fen:D-Fen	D-Nor-Fen ($ng\ ml^{-1}$)	D-Fen ($ng\ ml^{-1}$)	RATIO D-Nor-Fen:D-Fen
Sham D-Fen (Day 8)	4.32 ± 0.43	1.18 ± 0.25	3.88 ± 0.37	119 ± 20	34 ± 7	3.67 ± 0.22
ADX D-Fen (Day 8)	9.33 ± 1.12 *** c	7.86 ± 1.78 ** c	1.44 ± 0.32 *** c	184 ± 25 * c	160 ± 39 ** c	1.32 ± 0.22 ***
ADX D-Fen + corticosterone (Day 8)	4.61 ± 0.63	0.55 ± 0.14	7.34 ± 0.89 !!!	72 ± 4	BLQ	-

This makes the levels of D-Fen and the ratio D-Nor-Fen:D-Fen even more exaggerated when compared to the other two groups. Serum levels of D-Nor-Fen were 60% and 40% sham-ADX D-Fen and ADX D-Fen levels respectively, only significantly lower than the ADX D-Fen levels ($p<0.001$). No animal in the ADX D-Fen plus corticosterone group displayed detectable levels of D-Fen, and no calculation of the D-Nor-Fen:D-Fen is performed for this group.

3.6.4. Brain:serum ratios of D-Fen and D-Nor-Fen in acute and chronic treatment, and corticosterone-replacement treatment.

Table 3.13 expresses the data shown in Tables 3.11. and 3.12. in terms of the brain:serum ratio of both D-Nor-Fen and D-Fen. The brain:serum ratios of D-Nor-Fen and brain:serum D-Fen between respective termination groups show no significant difference (the value for D-Fen in corticosterone replacement cannot be calculated).

The ratio of (brain D-Nor-Fen:serum D-Nor-Fen):(brain D-Fen:serum D-Fen) is also shown in Table 3.13. The ratio of these two ratios shows similarity between respective sacrifice day animals, with day 7, day 51 and day 8 ratios of 1.12:1.12, 0.83:0.95 and 1.06:1.07 respectively for sham-ADX D-Fen:ADX D-Fen.

No significant difference in these ratios occurs between groups at any sacrifice time and to other sacrifice days.

Table 3.13. Brain:Serum ratios of D-Nor-Fen and D-Fen and the ratio Brain:serum [D-Nor-Fen:D-Fen] for the data presented in tables 3.11 and 3.12 (section 3.1 and 3.4).

Data points are means ± SEM.

No significance difference, between corresponding groups, is seen for any of the measured ratios.

Treatment	RATIO (Brain:Serum) D-Nor-Fen	RATIO (Brain:Serum) D-Fen	RATIO (Brain:Serum) D-Nor-Fen:D-Fen
Sham D-Fen (Day 7)	51.1 ± 4.4	45.7 ± 2.8	1.12 ± 0.07
ADX D-Fen (Day 7)	62.6 ± 4.7	56.0 ± 2.5	1.12 ± 0.08
Sham D-Fen (Day 51)	62.1 ± 3.1	72.9 ± 3.6	0.83 ± 0.04
ADX D-Fen (Day 51)	59.8 ± 2.6	62.9 ± 1.9	0.95 ± 0.04
Sham D-Fen (Day 8)	39.0 ± 6.4	37.8 ± 6.4	1.06 ± 0.10
ADX D-Fen (Day 8)	56.8 ± 12.0	51.4 ± 7.6	1.07 ± 0.13
ADX D-Fen + corticosterone (Day 8)	65.1 ± 9.7	- *	- *

* cannot be calculated

3.6.5. Correlation of D-Nor-Fen and D-Fen levels to parameters measured in sections 3.1 to 3.5.

The change in the body weight from day 1 to 7 (BW1), for the short-term animals correlates highly with levels of both brain and serum D-Nor-Fen and D-Fen:

- BW1 to brain D-Nor-Fen $r = -0.9326$ ($p<0.001$)
- BW1 to brain D-Fen $r = -0.9399$ ($p<0.001$)
- BW1 to ratio D-Nor-Fen:D-Fen $r = 0.8631$ ($p<0.01$).

Correlations to the levels seen in the serum show similar degrees of significance.

The food intake for the period day 1 to 7 also highly correlates with:

- Food intake to brain D-Nor-Fen $r = -0.9616$ ($p<0.001$)
- Food intake to brain D-Fen $r = -0.8814$ ($p<0.01$).

Correlations to the levels seen in the serum show similar degrees of significance.

No correlation between D-Nor-Fen or D-Fen levels is seen between serum levels of glucose or insulin.

Weights of liver and ovarian fat mass correlate to levels of brain D-Nor-Fen and D-Fen:

- $r = -0.9237$ ($p<0.01$) and $r = -0.9305$ ($p<0.01$) for liver weights
- $r = -0.902$ ($p<0.01$) and $r = -0.8369$ ($p<0.01$) for ovarian fat masses.

The only significant correlation in hypothalamic and hippocampal 5-HT, 5-HIAA, the ratio of the two and tryptophan occurs between hypothalamic 5-HT and the brain ratio of D-Nor-Fen:D-Fen.

The efficiency of food deposition (GEEFD) also highly correlates to the levels of D-Nor-Fen and D-Fen (and the ratio D-Nor-Fen:D-Fen) in both the brain and serum:

- GEEFD to brain D-Nor-Fen $r = -0.9498$ ($p<0.001$)
- GEEFD to brain D-Fen $r = -0.9200$ ($p<0.001$)
- GEEFD to ratio D-Nor-Fen:D-Fen $r = 0.7969$ ($p<0.01$).

Correlations to the levels seen in the serum are similarly significant.

By day 51, no significant correlations between the levels of D-Nor-Fen and D-Fen to any of the measured parameters are seen.

The D-Fen treated animals in the corticosterone-replacement experiment display identical profiles to the animals sacrificed on day 7 in terms of correlations of levels of brain and serum D-Nor-Fen and D-Nor-Fen to change in body weight and total food intake for day 1 to 8, and ovarian fat masses and hypothalamic 5-HT.

3.6.6. Discussion.

The levels of D-Nor-Fen, D-Fen and the ratio D-Nor-Fen:D-Fen in day 7, and the recovery of these by day 51 in the ADX D-Fen animals fit the profile exhibited in terms of body weight, food intake, the hypothalamic 5-HT system and the actions on the hypothalamic CRF levels with high correlates to body weight changes and total food intake to the levels of D-Nor-Fen and D-Fen experienced by the individual animals.

Adrenalectomy would appear to enhance the effect of D-Fen (and D-Nor-Fen) by prolonging its actions by maintaining brain and serum levels. The adrenal factor removed that is responsible for this effect would appear to be corticosterone. These results are discussed further in Chapter 4.

Chapter 4.

Discussion.

The paper by Arase *et al.* (1989c) demonstrated that adrenalectomy prevented the restoration of normal food intake observed with D,L-Fenfluramine and increased body weight loss and GDP binding to interscapular brown adipose tissue, in female Sprague-Dawley rats. The experimental design of the above study was such that animals were sacrificed 8 days following adrenalectomy.

When I repeated the experiment of Arase *et al.* and the animals allowed to survive for longer than 8 days, a reversal of these effects was observed. This thesis describes the results of a project designed to investigate the interaction of D-Fenfluramine with adrenal glucocorticoids in the regulation of food intake and energy balance, to establish possible loci and mechanism(s) of the interaction, and evaluate the results in terms of possible value for clinical usage.

The results presented in this thesis are a culmination of over 3 years work to eventually prove to one of the co-authors of the above paper that prolonging the experimental period produced a reversal in the body weight profile and anorexia. To account for the observation, and finally present the results in this thesis, the action of D, L-Fen, D-Fen and L-Fen was investigated in male and female Wistar rats, to show that the effects demonstrated by Arase *et al.* (1989c) were eventually reversed.

The terms "*acute*" and "*chronic*" D-Fen treatment mentioned in this discussion refer to my two basic experimental designs. The term "*acute*" D-Fen treatment refers to those animals sacrificed on days 7 (Section 3.1-3.3) or 8 (section 3.4), and is comparable to the study period applied by Arase *et al.*, whilst the term "*chronic*" treatment refers to those animals allowed to continue beyond the period demonstrated by Arase *et al.* (1989c) and sacrificed on day 51 (section 3.1-3.3).

At the end of each section in Chapter 3 (sections 3.1 - 3.6) the results were discussed briefly. All these results are summarised in the following two tables.

- Table 4.1. (page 169) summarises the results (compared to sham-ADX saline controls) of the acute and chronic experiments (section 3.1), the body composition analysis of these animals (section 3.3), the hypothalamic and hippocampal serotoninergic system (section 3.2), the hypothalamic CRF levels (section 3.5) and the brain/serum levels of D-Fen and D-Nor-Fen (section 3.6).

- Table 4.2. (page 170) summarises the results (compared to sham-ADX saline controls) of the corticosterone-replacement study (acute period), (section 3.4), including the hypothalamic and hippocampal serotoninergic system and the brain/serum levels of D-Fen and D-Nor-Fen (section 3.6)

It can be seen that the body weight profiles (acute and chronic period) for sham-ADX D-Fen animals were typical of the body weight changes seen by other investigators, i.e. an initial weight loss relative to controls, followed by restoration of the normal growth rate but at a trajectory below that of controls (sham-ADX saline) of approximately 40g after 51 days of administration (Innes *et al.* 1977, Duhault *et al.* 1979, Stunkard 1981, Douglas *et al.* 1983, Brindley *et al.* 1985, Rowland and Carlton 1986b). This action is not thought to be a reflection of tolerance to the actions of D-Fen, but possibly to metabolic adaptations to the lower weight.

The recovery in food intake to control levels by 3 days after D-Fen initiation was typical of the food intake profiles demonstrated by other investigators. Tolerance to the actions of Fen (and D-Fen) were observed by other investigators within 2 to 5 days, using various feeding paradigms including food deprivation schedules, dessert test or tail-pressure eating (Duhault *et al.* 1979, Brindley 1985, Fantino *et al.* 1986, 1988, Antelman *et al.* 1981, Curtis-Prior and Prouteau 1983, Heffer and Seiden 1979, Goudie *et al.* 1974, Rowland *et al.* 1982, Carlton and Rowland 1984, Antelman *et al.* 1981).

Table 4.1 Acute and Chronic treatment experiments

Comparison to control (Sham-ADX saline). Rounded to nearest 5%.

	D-Fen only		ADX only		D-Fen and ADX	
	Acute phase	Chronic phase	Acute phase	Chronic phase	Acute phase	Chronic phase
Body weight profile	- 10g [-15g]	-30g	-10g [-10g]	-20g	-25g [-30g]	-40g
Total food intake	75% [80%]	95%	75% [80%]	90%	50% [55%]	85%
Serum glucose	No change	95%	95%	90%	85%	90%
Serum insulin	115%	85%	60%	55%	50%	65%
Liver weight	85%	95%	80%	85%	60%	85%
Ovarian fat weight	75%	80%	35%	25%	15%	30%
Total body protein	95%	No change	95%	105%	80%	105%
Total body fat	85%	85%	70%	60%	75%	50%
Total body energy	No change	90%	85%	75%	70%	70%
HYPOTHALAMIC						
5-HT	45%	65%	110%	No change	20%	65%
5-HIAA	60%	65%	110%	105%	45%	75%
5-HT:5-HIAA	80%	No change	80%	No change	35%	95%
Tryptophan	90%	145%	No change	125%	95%	120%
HIPPOCAMPAL						
5-HT	25%	20%	75%	115%	5%	40%
5-HIAA	40%	35%	85%	130%	20%	65%
5-HT:5-HIAA	90%	50%	85%	90%	70%	60%
Tryptophan	105%	120%	95%	130%	80%	135%
Hypothalamic CRF	85%	NP	145%	NP	40%	NP
BRAIN						
D-Nor-Fen	100%*	100%**	N/A	N/A	265%	No change
D-Fen	100%*	100%**	N/A	N/A	535%	75%
D-Nor-Fen:D-Fen	100%*	100%**	N/A	N/A	55%	130%
SERUM						
D-Nor-Fen	100%*	100%**	N/A	N/A	170%	105%
D-Fen	100%*	100%**	N/A	N/A	290%	75%
D-Nor-Fen:D-Fen	100%*	100%**	N/A	N/A	70%	135%

Bracketed figures [body weight profile/total food intake] - chronic phase values at acute phase time point

NP Not performed * Acute phase control ** Chronic phase control

N/A not applicable (saline-treated group)

Table 4.2 Corticosterone-replacement experiment

Comparison to control (Sham-ADX saline). Rounded to nearest 5%.

	D-Fen only	ADX only	ADX (+corticosterone)	D-Fen and ADX	D-Fen and ADX (+corticosterone)
Body weight profile	-15g	-5g	No change	-35g	-15g
Food intake	80%	80%	90%	55%	80%
Serum glucose	No change	95%	No change	95%	No change
Serum insulin	115%	70%	85%	75%	95%
Liver weight	90%	85%	95%	65%	90%
Ovarian fat weight	60%	40%	70%	25%	65%
HYPOTHALAMIC					
5-HT	35%	80%	60%	20%	20%
5-HIAA	55%	No change	65%	50%	50%
5-HT:5-HIAA	70%	85%	90%	30%	55%
Tryptophan	125%	150%	155%	110%	145%
HIPPOCAMPAL					
5-HT	20%	85%	75%	50%	35%
5-HIAA	35%	105%	65%	30%	45%
5-HT:5-HIAA	65%	80%	120%	165%	95%
Tryptophan	No change	120%	145%	145%	135%
BRAIN					
D-Nor-Fen	100%*	N/A	N/A	215%	105%
D-Fen	100%*	N/A	N/A	665%	45%
D-Nor-Fen:D-Fen	100%*	N/A	N/A	35%	190%
SERUM					
D-Nor-Fen	100%*	N/A	N/A	155%	60%
D-Fen	100%*	N/A	N/A	470%	(BLQ)
D-Nor-Fen:D-Fen	100%*	N/A	N/A	35%	- **

* Sham-ADX D-Fen control values

N/A Not applicable (saline-treated groups)

BLQ Below limit of quantification

** Cannot be calculated

Adrenalectomy produces anorexia and an increase in energy expenditure, in rodents, by the transference of energy away from storage mechanisms to energy wastage by the mitochondria of brown adipose tissue. The anorexia produced by adrenalectomy, in these studies, was maintained at a body weight approximately 20g below sham-ADX controls, in agreement with other investigators.

The normal feeding pattern, in rodents, is to eat during the dark cycle (typically 1900-0700 hours), with minimal feeding during the light cycle. The restoration of feeding observed in ADX D-Fen animals at day 10 does not reflect a change of feeding (light and dark feeding measured) to the light cycle (when the levels of D-Fen and D-Nor-Fen would be expected to be lower).

The anomaly in ovarian fat masses between adrenalectomised and sham-ADX-operated animals after acute and chronic treatment, compared to their body weight profiles (see section 3.3), can possibly be explained by the lipid mobilisation effect of glucocorticoids. Glucocorticoids are known to promote the mobilisation of fat stores and cause specific relocation (as in Cushing's syndrome where fat distribution is seen to be localised around shoulders and face). The acute and chronic treatment total body lipids was reduced in all adrenalectomised animals compared to sham-ADX animals. The large difference in ovarian fat mass in the ADX D-Fen treatment animals compared to ADX saline animals was not reflected in the total lipid content of these animals, with:

sham-ADX saline > sham-ADX D-Fen > ADX saline = ADX D-Fen

To correlate the changes in body weight, body composition was studied with a view to understanding the relationship between anorexia and body set point and any differential effects on protein and lipid mobilisation and/or the efficiency of food deposition following the combination of D-Fen and adrenalectomy compared to either treatment alone. Acute and chronic treatment sham-ADX D-Fen animals showed slight reductions in total lipid compared to sham-ADX saline (as observed in the ovarian fat masses). The chronic treatment profiles of total lipid reflect the ovarian fat masses seen at this time in all treatment groups. The reduction in total body lipid confirms the observations by other investigators of fenfluramine decreasing the deposition of fat in epidydymal pads (Brindley *et al.* 1985) and the reduction in body fat more markedly than protein and muscle in animals (Beyen *et al.* 1986, Fantino and Faion 1986).

The acute ADX D-Fen treatment animals show a reduction in total body protein, which is a reflection of the lower body weight of the individuals, with both the acute sham-ADX D-Fen and ADX saline treatment animals showing small reductions (non-significant) compared to sham-ADX saline animals (again a reflection in the reduced body weight and the expected reduced lean body mass). Chronic treatment animals all have similar body protein levels, confirming the results shown by Beyen *et al.* 1986, Fantino and Faion 1986 in the reduction of body fat more markedly than protein and muscle.

Total body energy of the animals reflects the changes in the body weight profiles and the lipid/protein status. The calorific value of fat is over ten times that of protein, meaning that smaller change in lipid than protein can affect total energy composition of an individual. The large reductions in lipid observed in adrenalectomised animals are reflected in the lower energy composition of these animals. The acute treatment ADX D-Fen animals have even further reduced total energy content compared to acute treatment ADX saline by the lower levels of protein in comparison. Acute D-Fen alone appears not to affect total body energy composition, but at day 51 the energy content is lower than the sham-ADX saline animals (although non-significant).

The acute period (day 1 to 7) is a short period in terms of the analysis of changes in the dynamic profile of fat and protein deposition and/or mobilisation, and because of this, changes in the body weight profiles of an animal may be difficult to interpret in terms of very small discrete changes in the protein/fat/energy content. Although it is obvious that adrenalectomy produced dramatic changes in lipid and energy content, and adrenalectomised D-Fen treated animals had reduced protein content. The non-significant changes in lipid, protein and energy in the sham-ADX D-Fen animals compared to the sham-ADX saline animals, even though their body weight profile clearly suggests a reduction in one or a combination of these components may be a reflection of the short time scale and the errors associated. The chronic study demonstrated that the changes in body weight mirrored the changes in body composition for all groups, during which period the errors would have been reduced.

If the changes in body weight for the periods 1 to 7 (acute period) and 1 to 51 (chronic period) are correlated to the total food intake (from tables 3.2. and 3.3.), then the following intake of food necessary for 1g unit increase in body weight is demonstrated:

Acute period

- sham-ADX D-Fen = 9.5g g body weight^{-1}
- ADX D-Fen = - 7.3g g body weight^{-1} *
- sham-ADX saline = 6.3g g body weight^{-1}
- ADX saline = 11g g body weight^{-1}

* (7.3g food associated with a reduction of 1g body weight)

The ADX D-Fen value is rather ambiguous but the others clearly demonstrate that the food intake necessary to increase the body weights of the sham-ADX D-Fen and ADX saline animals is higher than the sham-ADX saline animals, and that the ADX D-Fen animals produced a net loss of body weight even with the intake shown.

The following intake of food necessary for 1g unit increase in body weight is demonstrated in the chronic study (day 1 to 51):

Chronic period

- sham-ADX D-Fen = 13g g body weight^{-1}
- ADX D-Fen = 13g g body weight^{-1}
- sham-ADX saline = 10g g body weight^{-1}
- ADX saline = 11g g body weight^{-1}

This shows that, for days 1 to 51, the sham-ADX D-Fen, ADX D-Fen and ADX saline animals consumed more food per unit increase in body weight compared to sham-ADX saline animals. The consumption of increased energy intake for unit changes in body weight suggests an increase in the ADX D-Fen, ADX saline and sham-ADX D-Fen individual's energy expenditure or a decrease in the utilisation of the energy from their diet. The values mirror the body weight profiles exhibited with sham-ADX saline > ADX saline > sham-ADX D-Fen = ADX D-Fen. These results confirm the observation

of other investigators in adrenalectomy and D-Fen increasing diet-induced thermogenesis (DIT) and increasing energy expenditure by stimulating thermogenesis and basal metabolic rate (Arase *et al.* 1989, Holt and York 1982, 1983 and Lupein and Bray 1985).

The gross energetic efficiency of food deposition (GEEFD) is very important. The evidence presented above is further supported by the GEEFD values. The calculation of the GEEFD is very similar to that of the values above for energy intake per unit change in body weight, with the exception that the intake of energy is correlated to the changes in total energy content of the animal by ballistic bomb calorimetry.

The acute treatment GEEFD values ($GEEFD_{day7}$), as would be expected, mirror the above results, with adrenalectomy producing a decreased utilisation of energy intake compared to sham-ADX animals (confirming the observations of other investigators) and the combination of adrenalectomy and D-Fen producing an even lower utilisation of energy intake at day 7. The lower $GEEFD_{day7}$ values suggest that the reductions in body weight associated with adrenalectomy are not singularly due to hypophagia but include an increased energy expenditure component. The further reduction of $GEEFD_{day7}$ in ADX D-Fen animals demonstrated a higher energy loss compared to adrenalectomy alone. The lower acute treatment body weights in sham-ADX D-Fen animals compared to sham-ADX saline is not reflected in a significant difference in $GEEFD_{day7}$, even though D-Fen is known to increase DIT and resting energy expenditure. This discrepancy may be due, in part, to the errors that may occur in such a short time period of the acute study.

Chronic treatment GEEFD values ($GEEFD_{day51}$) of both adrenalectomised animal groups are similar (although the ADX saline value is slightly lower than ADX D-Fen) at approximately 50% sham-ADX saline levels, with the $GEEFD_{day51}$ of sham-ADX D-Fen slightly lower than the sham-ADX saline animals (non-significant).

The acute and chronic treatment GEEFD values ($GEEFD_{day7}$ and $GEEFD_{day51}$) demonstrate that there is a recovery in the efficiency in food utilisation in ADX D-Fen

animals in line with the recovery in body weight, food intake and the hypothalamic 5-HT system, with both adrenalectomised GEEFD values still below that of sham-ADX animals. This supports the evidence that the removal of endogenous glucocorticoids by bilateral adrenalectomy increases energy wastage by brown adipose tissue. D-Fen alone appears to have no effect on the GEEFD values ($GEEFD_{day7}$ and $GEEFD_{day51}$), suggesting that, from this data, that the actions of D-Fen in reducing body weight are solely a result of a reduction in food intake.

Adrenalectomy reduced both insulin and glucose. The reduction in glucose levels by adrenalectomy may be attributable to a loss of hepatic-stimulated glucose production and the removal of glucocorticoid inhibition of glucose uptake. Insulin levels were decreased by the removal of glucocorticoid stimulation of the PSNS. This underlines the importance of glucocorticoid effects on insulin secretion. It would be expected that a lowering of serum insulin would cause an increase in glucose levels not the reduction observed, suggesting the glucocorticoid effects on glucose production was greater than insulin secretion.

The trend for sham-ADX D-Fen animals to display higher corticosterone levels (acute and chronic treatment) than sham-ADX saline animals (although non-significant) may be a reflection of the stimulation of the HPA axis by D-Fen. The levels of corticosterone, at the time of sacrifice (1100-1400 hours), would be expected to be low, and together with the reduced levels of D-Fen and D-Nor-Fen at this time (18 hours following injection) make the interpretation of the results difficult. Initially, D-Fen would stimulate the HPA and produce elevated corticosterone levels compared to sham-ADX saline animals, but this effect would only be pronounced as long as the levels of D-Fen were maintained. So, although the results suggest a non-significant effect of D-Fen on corticosterone levels, at the time of sacrifice, the early response to D-Fen should be considered.

The hypothalamic and hippocampal serotoninergic systems were investigated with a view to investigate if the actions of D-Fen or adrenalectomy singularly on these central 5-HT systems were modified by the combination of treatments. Both acute and chronic D-Fen

treatment levels of hypothalamic and hippocampal 5-HT and 5-HIAA were reduced. This data supports observations of other investigators (Kleven and Seiden 1989, Zaczek *et al.* 1990, Rowland 1986). Acute D-Fen treatment (sham-ADX and ADX) similarly caused a reduction in the ratio 5-HT:5-HIAA in both the hypothalamus and hippocampus, which has also been demonstrated by other groups (Fuller *et al.* 1978, Orosco *et al.* 1984, Rowland 1986).

The acute treatment levels of hypothalamic 5-HT, 5-HIAA and the ratio 5-HT:5-HIAA were further reduced in ADX-Fen treatment animals compared to sham-ADX D-Fen. Chronic D-Fen administration produced a reversal of the 5-HT depletion and reduced ratio 5-HT:5-HIAA observed in the ADX D-Fen group compared to their sham-ADX D-Fen controls, although the 5-HT levels in both D-Fen treatment groups were maintained significantly lower than both saline-treated groups.

Adrenalectomy alone had no effect on the hypothalamic 5-HT profile. Neither D-Fen nor adrenalectomy alone had any effects on hypothalamic tryptophan. Others have demonstrated adrenalectomy to have either no effect on brain 5-HT (Shah *et al.* 1968) or a reduction (Kovács *et al.* 1977, Rastogi and Singhal 1978, Telegdy and Vermes 1975). The changes in 5-HT (where occurring) are not apparent until one week following adrenalectomy (Rastogi and Singhal 1978).

The acute treatment levels of hippocampal 5-HT, 5-HIAA and the ratio 5-HT:5-HIAA were further reduced in ADX-Fen treatment animals compared to sham-ADX D-Fen (as seen in the hypothalamus). Chronic D-Fen administration produced a reversal of the 5-HT depletion and reduced ratio 5-HT:5-HIAA observed in the ADX D-Fen group compared to their sham-ADX D-Fen controls. However, unlike the hypothalamus, the ratio 5-HT:5-HIAA in both the chronic D-Fen treatment groups is maintained at a significant level below that of the saline controls.

Adrenalectomy alone had no effect on the hippocampal 5-HT profile. Other investigators have demonstrated that corticosteroids reduces 5-HT and 5-HIAA in rat hippocampus (De Kloet *et al.* 1983, Balfour *et al.* 1979) and not the hypothalamus

(Balfour *et al.* 1979). Adrenalectomy produced a decrease and increase in acute and chronic treatment hippocampal tryptophan levels respectively. In confirmation, Miller *et al.* 1978 demonstrated increases in brain tryptophan following long-term adrenalectomy. D-Fen had no effect on hippocampal tryptophan (also demonstrated [brain levels] by Costa *et al.* 1971).

Confirmation of the adrenal factor responsible for the potentiation of the effects of D-Fen was investigated by replacement corticosterone therapy to adrenalectomised D-Fen animals (results presented in section 3.4). The final end point body weight of the ADX D-Fen plus corticosterone and the ADX saline plus corticosterone groups (section 3.4), (at day 8 instead of day 7 as with the previous acute study), were restored to their respective sham-ADX animal controls (sham-ADX D-Fen and sham-ADX saline respectively). However, the body weight profiles are slightly different. The profile of the ADX saline plus corticosterone group mirrors that of the sham-ADX saline animals, but the profile of the ADX D-Fen plus corticosterone animals is off-set by a fall in body weight following surgery which is greater than that seen with the sham-ADX D-Fen animals, and takes until the end of the study to be restored to the sham-ADX D-Fen level. The significance of this is discussed later.

Corticosterone replacement restored serum glucose and insulin to their respective sham-ADX controls, and restored fully the reductions observed in liver and ovarian fat masses associated in the two adrenalectomy groups. The only discrepancy in the total food intake occurred in the ADX saline plus corticosterone animals. This group had a significantly lower total food intake compared to the sham-ADX saline group and may be a reflection of the lower pre-surgical levels in the corticosterone replacement animals and/or the failure to reproduce exactly the sham-ADX-operated conditions immediately following surgery in terms of the physiological levels of plasma corticosterone.

The decrease in the hypothalamic 5-HT profile associated with adrenalectomy in D-Fen treated animals was partially restored to the sham-ADX D-Fen profile by the replacement of corticosterone. This suggests that the adrenal factor removed that is

responsible for the reduction of hypothalamic 5-HT and the reduction in the ratio of 5-HT:5-HIAA ratio in ADX D-Fen animals compared to sham-ADX D-Fen is corticosterone. Less information can be obtained from the hippocampal data because of the large variation associated with 5-HT, and the large 5-HT:5-HIAA ratio. Other investigators have demonstrated that corticosteroids reduce 5-HT and 5-HIAA in rat hippocampus (De Kloet *et al.* 1983, Balfour *et al.* 1979) and not the hypothalamus (Balfour *et al.* 1979). Others have demonstrated adrenalectomy to have either no effect on brain 5-HT (Shah *et al.* 1968) or a reduction (Kovács *et al.* 1977, Rastogi and Singhal 1978, Telegdy and Vermes 1975). The changes in 5-HT (where occurring) were not apparent until one week following adrenalectomy (Rastogi and Singhal 1978).

No effect of surgery (adrenalectomy), D-Fen or corticosterone replacement on either hypothalamic or hippocampal tryptophan was observed.

The failure to completely abolish the effect of adrenalectomy in the ADX D-Fen animals by corticosterone replacement, could be a reflection of the lack of normal adrenal corticosterone secretion for the initial period of adrenalectomy. This is hypothesised to be a result of the time taken for the arachis oil preparation to release corticosterone and raise plasma levels, for the period immediately following surgery, to a physiological level.

The failure of corticosterone replacement to, in the initial stages, restore adrenalectomised animal's food intake profiles to sham-ADX levels further suggests that the corticosterone pool in arachis oil does not compensate fully for the normal endogenous corticosterone secretion.

In hindsight, it would probably have been more appropriate to begin corticosterone replacement some time before the animals were adrenalectomised. The corticosterone was administered immediately following the adrenalectomy of the animals and therefore the surgical- and anaesthetic-induced increases in the HPA axis activity and associated increases in adrenal corticosterone (and noradrenaline) levels would not be seen by these animals. Therefore, for a short period, the corticosterone-replacement-

adrenalectomised animals will, in theory, have experienced a period of "*adrenalectomy*" until the arachis oil pool increased blood levels of corticosterone to a physiological level. The blood levels of corticosterone would appear to be resumed to normal levels by the observation that ADX D-Fen plus corticosterone food intake was restored to sham-ADX D-Fen levels by day 4.

The immunocytochemistry localisation of CNS CRF provided no positive data into the interpretation of the previous results, but appeared, at the time, a logical step to investigate the possible loci and mechanism of the interaction of adrenalectomy and D-Fen.

IRMA detection of CRF, however, allowed hypothalamic CRF levels to be measured quantitatively. The CRF levels like 5-HT, however, give an indication of release activity. The lower levels of CRF are taken that stimulation of release has occurred and presented the animal with an increased exposure to the actions of CRF and a reflection of the inability to compensate for the increased release by CRF synthesis. D-Fen has been demonstrated to significantly lower CRF levels specifically in the hypothalamus, with no significant change in any other regions excepting small increases in the hippocampus, midbrain and spinal cord (Appel *et al.* 1991). Appel *et al.* (1991) also demonstrated that the tolerance to the actions of chronic D-Fen administration may be associated with a reduction in the responsiveness of the HPA axis. Chronic D-Fen treated animals show significant reductions in the stimulation of corticosterone production compared to acute treatment. The tolerance may be due, in part, to the lower pool of available 5-HT and/or CRF for the production of the D-Fen effects on body weight, food intake and energy expenditure. Also, as with 5-HT, consideration of the extent (if any) of super-sensitivity needs to be considered in the interpretation of long-term studies producing alteration in the levels of a neurotransmitter (5-HT or CRF in this case).

The increase of hypothalamic CRF observed, in the adrenalectomised saline-treated animal group (ADX saline), is postulated to be a result of the loss of negative feedback inhibition by corticosterone (also observed by other investigators). The reduction in

hypothalamic CRF, by D-Fen, is taken as an index of stimulation of CRF release from CRF-containing cells, by the depletion of storage without replenishment.

The reduced hypothalamic CRF in ADX D-Fen animals compared to either sham-ADX D-Fen or ADX saline animals may be due to the increased hypothalamic 5-HT activity associated with these animals (see section 3.2.), causing an increase in CRF release compared to the sham-ADX D-Fen animals.

The results clearly demonstrate that increases in hypothalamic CRF content associated with adrenalectomy are reduced with D-Fen treatment. The non-significant reduction of hypothalamic CRF in sham-ADX D-Fen animals compared to sham-ADX saline may be a reflection of the lower 5-HT activity in the sham-ADX D-Fen animals compared to ADX D-Fen animals. Simply, it may be hypothesised, that the stimulation of CRF release by 5-HT is greater in the ADX D-Fen animals compared to sham-ADX D-Fen purely by the increase in 5-HT activity associated with these animals. The fact that ADX D-Fen CRF levels (approximately 8 ng g^{-1}) are lowered below that of the sham-ADX D-Fen levels (approximately 20ng g^{-1}) from the high levels associated with adrenalectomy (approximately 30ng g^{-1}) suggests an even higher CRF release by D-Fen in adrenalectomised animals, and so promoting increases in CRF actions in inhibiting food intake and stimulating the sympathetic nervous system.

The failure of D-Fen to reduce CRF levels in sham-ADX D-Fen animals is, as mentioned previously, probably a result of lowered 5-HT activity. Appel *et al.* (1991), however, demonstrate a 50% reduction in hypothalamic CRF in animals receiving 24mg kg^{-1} Fen twice daily for 4 days. Acute treatment, by this group, showed no significant lowering of hypothalamic CRF and may have been the result of replenishment of CRF by synthesis in the hypothalamus (Berkenbosch and Tilders 1988, Berkenbosch *et al.* 1989).

The results of the measurement of hypothalamic CRF levels clearly show that the potentiation of body weight loss and hypophagia in ADX D-Fen animals, compared to either treatment alone, is associated with an increase in the activity of hypothalamic

corticotrophin-releasing factor. However, the complete dynamics of the CRF system may not be completely interpreted from this data because of possible changes in receptor numbers, CRF synthesising dynamics and/or changes in cell populations producing CRF.

The results that are clearly of most importance are the measurements of D-Fen and D-Nor-Fen performed on all the D-Fen treated animals (presented in section 3.1-3.4).

Acute treatment ADX D-Fen (section 3.1-3.3) animals demonstrated significantly elevated brain D-Nor-Fen and D-Fen levels of 2.6 and 5.5 fold ($p<0.001$ in both cases) compared to sham-ADX D-Fen animals, with the ratio of D-Nor-Fen:D-Fen in the ADX D-Fen significantly lowered at 50% of sham-ADX D-Fen levels ($p<0.05$). Serum levels of D-Nor-Fen and D-Fen were similar to brain levels, with a significant 1.7 and 2.9 fold increase respectively of D-Nor-Fen and D-Fen ($p<0.01$ and $p<0.05$ respectively) in ADX D-Fen animals compared to sham-ADX D-Fen animals, and a significant reduction of the D-Nor-Fen:D-Fen ratio to 70% of sham-ADX D-Fen levels ($p<0.05$).

Chronic treatment ADX D-Fen animals demonstrated levels of both brain and serum D-Nor-Fen and D-Fen that were non-significantly different from sham-ADX D-Fen animals. The ratio of D-Nor-Fen:D-Fen was similarly non-significant to sham-ADX D-Fen animals. The trend for acute treatment ADX D-Fen animals to display increased D-Fen and D-Nor-Fen and a reduced D-Nor-Fen:D-Fen ratio (brain and serum) seems to have been reversed.

A similar trend for the acute animals, section 3.4. (corticosterone replacement experiment), was seen in terms of the brain D-Nor-Fen and D-Fen levels, with a significant 2.2 and 6.7 fold increase respectively in the ADX D-Fen animals compared to sham-ADX D-Fen animals ($p<0.001$ and $p<0.01$ respectively). The ratio D-Nor-Fen:D-Fen was also similarly significantly 40% lower in the ADX D-Fen animals compared to sham-ADX D-Fen animals ($p<0.001$). Serum levels of D-Nor-Fen and D-Fen were also similarly significantly elevated by 1.5 and 4.7 fold respectively in

ADX D-Fen animals compared to sham-ADX D-Fen ($p<0.05$ and $p<0.01$ respectively). The ratio D-Nor-Fen:D-Fen was similarly significantly 36% of the sham-ADX D-Fen level ($p<0.001$).

Corticosterone replacement provided some interesting results. Brain D-Nor-Fen and D-Fen levels were significantly lower in the ADX D-Fen plus corticosterone animals compared to ADX D-Fen animals ($p<0.001$ in both cases), and non-significantly different from sham-ADX D-Fen levels (although D-Fen was 50% sham-ADX D-Fen levels). The ratio D-Nor-Fen:D-Fen was significantly 1.9 and 5.1 fold higher in the ADX D-Fen plus corticosterone animals compared to sham-ADX D-Fen and ADX D-Fen respectively ($p<0.001$ in both cases). One animal in the ADX D-Fen plus corticosterone displayed non-detectable levels of brain D-Fen, and was not included in the D-Fen table and subsequent D-Nor-Fen:D-Fen analysis. This makes the levels of D-Fen and the ratio D-Nor-Fen:D-Fen even more exaggerated when compared to the other two groups. Serum levels of D-Nor-Fen were 60% and 40% sham-ADX D-Fen and ADX D-Fen levels respectively, only significantly lower than the ADX D-Fen levels ($p<0.001$). No animal in the ADX D-Fen plus corticosterone group displayed detectable levels of serum D-Fen, and therefore no calculation of the serum D-Nor-Fen:D-Fen ratio is possible for this study group.

Rowland and Carlton, 1986b, indicate that brain levels of D-Fen and D-Nor-Fen, in rodent animal studies (although only single injection figure are quoted), are often about 10-40 fold higher than the corresponding plasma levels. In confirmation, the results presented in table 3.13 demonstrate brain values of between x40 and x70 serum levels (both D-Fen and D-Nor-Fen).

The brain:serum ratios of D-Nor-Fen and brain:serum D-Fen between respective termination groups show no significant difference (the value for D-Fen in corticosterone replacement cannot be calculated), although the trend in the early sacrifice days (day 7 and 8) is for the adrenalectomised animals (ADX D-Fen) to have increased brain:serum ratio's for both D-Nor-Fen and D-Fen, suggesting a slight

increase in the brain uptake for D-Nor-Fen and D-Fen and/or an inhibition of the processes governing the removal of D-Nor-Fen and D-Fen from the brain.

The "*ratio of these two ratios*" shows similarity between respective sacrifice day animals, with acute (day 7), chronic (day 51) and acute (day 8) ratios of 1.12:1.12, 0.83:0.95 and 1.06:1.07 respectively for sham-ADX D-Fen:ADX D-Fen. No significant difference in these ratios occurs between groups at any sacrifice time and to other sacrifice days. The significance of this is discussed later.

At the acute treatment point (day 7 or day 8), it would appear that adrenalectomy removed some mechanism responsible for the metabolism of D-Fen to D-Nor-Fen and the removal of both from the brain and serum. At the chronic treatment point (day 51), it would appear that this effect has been restored to sham-ADX levels.

The corticosterone-replacement data suggests that the replacement of corticosterone to adrenalectomised animals restored a previously inhibited metabolic pathway for D-Fen and thus restoring D-Fen and D-Nor-Fen to the levels seen in sham-ADX D-Fen animals. In fact, corticosterone-replacement, in these animals would appear to have increased metabolism, which may be a result of over-stimulation of the metabolic process of D-Fen elimination (compared to sham-ADX D-Fen animals). The reduced levels of brain and serum D-Nor-Fen and D-Fen in the corticosterone-replacement animals (ADX D-Fen plus corticosterone) compared to sham-ADX D-Fen animals would appear, at first, not to be associated with higher levels of corticosterone. At termination, the levels of serum corticosterone are in the order sham-ADX D-Fen > sham-ADX saline > ADX saline plus corticosterone > ADX D-Fen plus corticosterone. This anomaly can possibly be explained by the profile of corticosterone exposure these animals would have experienced. Corticosterone-replacement animals, as previously mentioned, received a corticosterone and arachis oil bolus injection (s.c.) at approximately 1800 hours, and the animals were sacrificed 18 to 20 hours later. The injection of corticosterone was given just before the start of the dark cycle in an attempt to mimic the rise in blood corticosterone associated before the dark cycle in sham-ADX operated rats.

The observation that ADX D-Fen plus corticosterone animals display lower levels of brain D-Nor-Fen and D-Fen and low levels of D-Nor-Fen (with no detectable D-Fen) suggests that the corticosterone dosage presented these animals with an initial "*hyper-exposure*" to corticosterone compared to their sham-ADX controls, which increased the metabolism of D-Fen. The lower levels of serum corticosterone in the ADX D-Fen plus corticosterone animals (and ADX saline plus corticosterone) could be attributed to an exhaustion of the corticosterone in the arachis oil pool and secretions of corticosterone at sub sham-ADX levels.

The fact that the brain:serum ratios of D-Nor-Fen and brain:serum ratio of D-Fen showed no significant difference suggests that surgery (adrenalectomy) does not affect the ability to sequestrate D-Nor-Fen and D-Fen into the brain, although the trend in the early sacrifice days (day 7 and 8) is for the adrenalectomised animals to have increased brain:serum ratio's for both D-Nor-Fen and D-Fen, suggesting a slight increase in the brain uptake for D-Nor-Fen and D-Fen and/or an inhibition of the processes governing the removal of D-Nor-Fen and D-Fen from the brain. This trend is absent at the chronic treatment stage. These ratios suggest that the uptake, deposition and metabolism of D-Nor-Fen compared to D-Fen are identical for adrenalectomy and sham-ADX surgery. A ratio of 1 for all the cases also suggests that surgery has no effect on the ability to sequester either D-Nor-Fen or D-Fen preferentially, and the high levels of D-Nor-Fen and D-Fen, together with the reduction of the ratio D-Nor-Fen:D-Fen, in ADX D-Fen animals suggests that adrenalectomy removes a process(es) responsible for the metabolism of D-Fen to D-Nor-Fen and elimination of both from the brain and serum.

Glucocorticoids are known to protect against the toxic effects of many drugs by inducing UDP-glucuronosyltransferase, NADPH-cytochrome P_{450} oxidoreductase and cytochromes P_{450} PCN. Cytochrome P_{450} PCN is the most sensitive to glucocorticoid induction. The mechanism of glucocorticoid (dexamethasone) induction is both transcriptional and post-transcriptional (message stabilisation), unlike phenobarbital and polycyclic aromatic hydrocarbons that induce the mixed-function oxidase system by stimulation of gene transcription. It is one of these stages that, I propose, is removed

following adrenalectomy to inhibit a pathway(s) for the metabolism of D-Fenfluramine (and D-Nor-Fenfluramine). The primary route of D-Fen and D-Nor-Fen metabolism is by hepatic enzymes.

High correlations between the levels of brain and serum D-Fen or D-Nor-Fen are seen for acute changes in body weight and food intake (section 3.1. and 3.6), $GEEFD_{day7}$ and the hypothalamic 5-HT profile. The acute D-Fen (day 8 sacrifice) treated animals in the corticosterone-replacement experiment display identical profiles to the animals sacrificed on day 7 in terms of correlations of levels of brain and serum D-Nor-Fen and D-Nor-Fen to change in body weight and total food intake for day 1 to 8 and hypothalamic 5-HT.

The acute levels of D-Nor-Fen, D-Fen and the ratio D-Nor-Fen:D-Fen and the recovery of these at the chronic phase treatment, in the ADX D-Fen animals, fit the profile exhibited in terms of body weight, food intake, the hypothalamic 5-HT system and the actions on the hypothalamic CRF levels.

Conclusion

In summary adrenalectomy, in female Wistar rats, would appear to enhance the effect of D-Fen (and D-Nor-Fen) by prolonging its actions by maintaining brain and serum levels. The adrenal factor removed that is responsible for this effect would appear to be corticosterone. The removal of corticosterone by adrenalectomy removes a metabolic process for the metabolism of D-Fen and/or the elimination of D-Nor-Fen.

The results presented in this thesis (sections 3.1 - 3.5) would appear to support the hypothesis of an increased exposure to the effects of D-Fen and D-Nor-Fen in adrenalectomised rats, namely increased body weight loss, reduction in food intake, decreased efficiencies of food deposition and body composition analysis, increased hypothalamic and hippocampal serotinergic system and hypothalamic CRF activity.

There are no reports of use of D-Fen in adrenal disease states or listings of contraindications/warnings against use in patients with corticosteroid insufficiency or concomitant administration with corticosteroids (Servier Medical Information Department February 1997, Adifax® datasheet, ABPI Compendium of Datasheets 1996-1997) or indicated in Drug Interactions, Fourth Edition (Ed. I.H. Stockley, The Pharmaceutical Press).

It is possible to conclude from my results that there would be a possible interaction of D-Fen and corticosteroids to produce a reduction in D-Fen efficacy by promoting metabolism of D-Fen and D-Nor-Fen.

It must always be borne in mind, however, that animal experiments only give an indication of the situation that may arise in humans. However, it is clear, from my results presented in this thesis, the interaction of D-Fen and glucocorticoids deserves further investigation.

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APPENDIX 1. (FIGURES: 2 way ANOVA F / p values)

Figure 3.1. Body weight profile to day 7. Page 94.

Adrenalectomy verses sham			D-Fen verses saline		
Day –2	$F_{1,24}$ =	1.02 (NS, p=0.325)	Day –2	$F_{1,24}$ =	0.37 (NS, p=0.552)
Day –1	$F_{1,24}$ =	0.01 (NS, p=0.932)	Day –1	$F_{1,24}$ =	0.61 (NS, p=0.444)
Day 0	$F_{1,24}$ =	0.37 (NS, p=0.553)	Day 0	$F_{1,24}$ =	0.03 (NS, p=0.957)
Day 1	$F_{1,24}$ =	2.36 (NS, p=0.140)	Day 1	$F_{1,24}$ =	3.82 (NS, p=0.065)
Day 2	$F_{1,24}$ =	0.56 (NS, p=0.462)	Day 2	$F_{1,24}$ =	24.96 (p<0.001)
Day 3	$F_{1,24}$ =	2.31 (NS, p=0.144)	Day 3	$F_{1,24}$ =	47.19 (p<0.001)
Day 4	$F_{1,24}$ =	15.72 (p=0.001)	Day 4	$F_{1,24}$ =	55.37 (p<0.001)
Day 5	$F_{1,24}$ =	20.96 (p<0.001)	Day 5	$F_{1,24}$ =	39.91 (p<0.001)
Day 6	$F_{1,24}$ =	26.44 (p<0.001)	Day 6	$F_{1,24}$ =	33.47 (p<0.001)
Day 7	$F_{1,24}$ =	29.28 (p<0.001)	Day 7	$F_{1,24}$ =	29.28 (p<0.001)
Interaction					
Day –2	$F_{1,24}$ =	11.75 (p=0.03)			
Day –1	$F_{1,24}$ =	8.19 (p=0.01)			
Day 0	$F_{1,24}$ =	2.54 (NS, p=0.127)			
Day 1	$F_{1,24}$ =	2.81 (NS, p=0.109)			
Day 2	$F_{1,24}$ =	1.26 (NS, p=0.274)			
Day 3	$F_{1,24}$ =	0.45 (NS, p=0.511)			
Day 4	$F_{1,24}$ =	0.36 (NS, p=0.557)			
Day 5	$F_{1,24}$ =	0.21 (NS, p=0.652)			
Day 6	$F_{1,24}$ =	0.52 (NS, p=0.478)			
Day 7	$F_{1,24}$ =	0.41 (NS, p=0.529)			

Figure 3.2. Food intake profile to day 7. Page 97.

Adrenalectomy verses sham			D-Fen verses saline		
Day –3	$F_{1,24}$ =	0.44 (NS, p=0.515)	Day –3	$F_{1,24}$ =	2.91 (NS, p=0.104)
Day –2	$F_{1,24}$ =	0.67 (NS, p=0.424)	Day –2	$F_{1,24}$ =	0.06 (NS, p=0.804)
Day –1	$F_{1,24}$ =	0.02 (NS, p=0.884)	Day –1	$F_{1,24}$ =	0.48 (NS, p=0.497)
Day 0	$F_{1,24}$ =	1.68 (NS, p=0.209)	Day 0	$F_{1,24}$ =	3.96 (NS, p=0.061)
Day 1	$F_{1,24}$ =	1.23 (NS, p=0.280)	Day 1	$F_{1,24}$ =	911.39 (p<0.001)
Day 2	$F_{1,24}$ =	0.37 (NS, p=0.553)	Day 2	$F_{1,24}$ =	38.50 (p<0.001)
Day 3	$F_{1,24}$ =	385.66 (p<0.001)	Day 3	$F_{1,24}$ =	41.14 (p<0.001)
Day 4	$F_{1,24}$ =	32.32 (p<0.001)	Day 4	$F_{1,24}$ =	1.68 (NS, p=0.293)
Day 5	$F_{1,24}$ =	26.00 (p<0.001)	Day 5	$F_{1,24}$ =	50.90 (p<0.001)
Day 6	$F_{1,24}$ =	55.07 (p<0.001)	Day 6	$F_{1,24}$ =	10.76 (p=0.004)
Interaction					
Day –3	$F_{1,24}$ =	3.84 (NS, p=0.064)			
Day –2	$F_{1,24}$ =	4.37 (p=0.05)			
Day –1	$F_{1,24}$ =	1.24 (NS, p=0.279)			
Day 0	$F_{1,24}$ =	0.13 (NS, p=0.720)			
Day 1	$F_{1,24}$ =	2.39 (NS, p=0.138)			
Day 2	$F_{1,24}$ =	0.63 (NS, p=0.435)			
Day 3	$F_{1,24}$ =	4.83 (p=0.04)			
Day 4	$F_{1,24}$ =	1.61 (NS, p=0.219)			
Day 5	$F_{1,24}$ =	1.23 (NS, p=0.281)			
Day 6	$F_{1,24}$ =	0.84 (NS, p=0.369)			

Figure 3.3. Body weight profile to day 51. Page 100.

Adrenalectomy verses sham			D-Fen verses saline		
Day –2	$F_{1,24}$ =	0.87 (NS, p=0.362)	Day –2	$F_{1,24}$ =	3.37 (NS, p=0.083)
Day –1	$F_{1,24}$ =	1.24 (NS, p=0.280)	Day –1	$F_{1,24}$ =	1.91 (NS, p=0.183)
Day 0	$F_{1,24}$ =	2.02 (NS, p=0.173)	Day 0	$F_{1,24}$ =	0.20 (NS, p=0.663)
Day 1	$F_{1,24}$ =	0.63 (NS, p=0.438)	Day 1	$F_{1,24}$ =	0.32 (NS, p=0577)
Day 2	$F_{1,24}$ =	0.06 (NS, p=0.940)	Day 2	$F_{1,24}$ =	5.25 (p=0.034)
Day 3	$F_{1,24}$ =	0.04 (NS, p=0.952)	Day 3	$F_{1,24}$ =	10.81 (p=0.004)
Day 4	$F_{1,24}$ =	1.28 (NS, p=0.273)	Day 4	$F_{1,24}$ =	20.03 (p<0.001)
Day 5	$F_{1,24}$ =	3.75 (NS, p=0.069)	Day 5	$F_{1,24}$ =	22.00 (p<0.001)
Day 6	$F_{1,24}$ =	5.06 (p=0.037)	Day 6	$F_{1,24}$ =	19.06 (p<0.001)
Day 7	$F_{1,24}$ =	10.40 (p=0.005)	Day 7	$F_{1,24}$ =	21.09 (p<0.001)
Day 8	$F_{1,24}$ =	17.49 (p=0.001)	Day 8	$F_{1,24}$ =	25.60 (p<0.001)
Day 9	$F_{1,24}$ =	16.09 (p=0.001)	Day 9	$F_{1,24}$ =	24.67 (p<0.001)
Day 10	$F_{1,24}$ =	16.73 (p=0.001)	Day 10	$F_{1,24}$ =	30.40 (p<0.001)
Day 11	$F_{1,24}$ =	14.63 (p=0.001)	Day 11	$F_{1,24}$ =	29.74 (p<0.001)
Day 12	$F_{1,24}$ =	13.65 (p=0.002)	Day 12	$F_{1,24}$ =	29.30 (p<0.001)
Day 13	$F_{1,24}$ =	9.77 (p=0.006)	Day 13	$F_{1,24}$ =	29.57 (p<0.001)
Day 14	$F_{1,24}$ =	8.02 (p=0.011)	Day 14	$F_{1,24}$ =	22.86 (p<0.001)
Day 15	$F_{1,24}$ =	6.07 (p=0.024)	Day 15	$F_{1,24}$ =	18.02 (p<0.001)
Day 16	$F_{1,24}$ =	3.85 (NS, p=0.065)	Day 16	$F_{1,24}$ =	15.11 (p=0.001)
Day 17	$F_{1,24}$ =	2.88 (NS, p=0.107)	Day 17	$F_{1,24}$ =	17.64 (p=0.001)
Day 18	$F_{1,24}$ =	3.94 (NS, p=0.063)	Day 18	$F_{1,24}$ =	17.87 (p=0.001)
Day 19	$F_{1,24}$ =	5.07 (p=0.037)	Day 19	$F_{1,24}$ =	13.92 (p=0.002)
Day 20	$F_{1,24}$ =	6.82 (p=0.018)	Day 20	$F_{1,24}$ =	11.33 (p=0.003)
Day 21	$F_{1,24}$ =	8.54 (p=0.009)	Day 21	$F_{1,24}$ =	18.83 (p<0.001)
Day 22	$F_{1,24}$ =	8.18 (p=0.010)	Day 22	$F_{1,24}$ =	13.83 (p=0.002)
Day 23	$F_{1,24}$ =	6.14 (p=0.023)	Day 23	$F_{1,24}$ =	11.96 (p=0.003)
Day 24	$F_{1,24}$ =	7.45 (p=0.014)	Day 24	$F_{1,24}$ =	11.41 (p=0.003)
Day 25	$F_{1,24}$ =	5.60 (p=0.029)	Day 25	$F_{1,24}$ =	12.80 (p=0.002)
Day 26	$F_{1,24}$ =	6.27 (p=0.022)	Day 26	$F_{1,24}$ =	12.09 (p=0.003)
Day 27	$F_{1,24}$ =	6.55 (p=0.020)	Day 27	$F_{1,24}$ =	9.04 (p=0.008)
Day 28	$F_{1,24}$ =	4.48 (p=0.049)	Day 28	$F_{1,24}$ =	8.93 (p=0.008)
Day 29	$F_{1,24}$ =	5.57 (p=0.030)	Day 29	$F_{1,24}$ =	9.43 (p=0.007)
Day 30	$F_{1,24}$ =	9.24 (p=0.007)	Day 30	$F_{1,24}$ =	18.27 (p<0.001)
Day 31	$F_{1,24}$ =	5.89 (p=0.026)	Day 31	$F_{1,24}$ =	17.85 (p=0.001)
Day 32	$F_{1,24}$ =	4.17 (NS, p=0.056)	Day 32	$F_{1,24}$ =	17.03 (p=0.001)
Day 33	$F_{1,24}$ =	3.95 (NS, p=0.062)	Day 33	$F_{1,24}$ =	14.01 (p=0.001)
Day 34	$F_{1,24}$ =	3.34 (NS, p=0.084)	Day 34	$F_{1,24}$ =	12.35 (p=0.002)
Day 35	$F_{1,24}$ =	3.85 (NS, p=0.065)	Day 35	$F_{1,24}$ =	12.24 (p=0.003)
Day 36	$F_{1,24}$ =	4.09 (NS, p=0.058)	Day 36	$F_{1,24}$ =	11.75 (p=0.003)
Day 37	$F_{1,24}$ =	4.40 (p=0.050)	Day 37	$F_{1,24}$ =	11.16 (p=0.004)
Day 38	$F_{1,24}$ =	4.70 (p=0.044)	Day 38	$F_{1,24}$ =	10.24 (p=0.005)
Day 39	$F_{1,24}$ =	4.79 (p=0.042)	Day 39	$F_{1,24}$ =	11.20 (p=0.004)
Day 40	$F_{1,24}$ =	5.04 (p=0.038)	Day 40	$F_{1,24}$ =	12.16 (p=0.003)
Day 41	$F_{1,24}$ =	5.28 (p=0.034)	Day 41	$F_{1,24}$ =	13.73 (p=0.002)
Day 42	$F_{1,24}$ =	4.66 (p=0.045)	Day 42	$F_{1,24}$ =	14.17 (p=0.001)
Day 43	$F_{1,24}$ =	4.52 (p=0.048)	Day 43	$F_{1,24}$ =	12.54 (p=0.002)
Day 44	$F_{1,24}$ =	4.67 (p=0.044)	Day 44	$F_{1,24}$ =	11.09 (p=0.004)
Day 45	$F_{1,24}$ =	3.81 (NS, p=0.067)	Day 45	$F_{1,24}$ =	10.93 (p=0.004)
Day 46	$F_{1,24}$ =	3.35 (NS, p=0.084)	Day 46	$F_{1,24}$ =	13.45 (p=0.002)
Day 47	$F_{1,24}$ =	2.49 (NS, p=0.132)	Day 47	$F_{1,24}$ =	13.18 (p=0.002)
Day 48	$F_{1,24}$ =	2.59 (NS, p=0.125)	Day 48	$F_{1,24}$ =	10.49 (p=0.005)
Day 49	$F_{1,24}$ =	2.28 (NS, p=0.149)	Day 49	$F_{1,24}$ =	11.43 (p=0.003)
Day 50	$F_{1,24}$ =	2.10 (NS, p=0.165)	Day 50	$F_{1,24}$ =	12.98 (p=0.002)
Day 51	$F_{1,24}$ =	2.18 (NS, p=0.157)	Day 51	$F_{1,24}$ =	15.11 (p=0.001)

Figure 3.3. Body weight profile to day 51. Page 100 (continued).

Interaction

Day −2	$F_{1,24}$ =	3.54	(NS, p=0.076)
Day −1	$F_{1,24}$ =	2.49	(NS, p=0.132)
Day 0	$F_{1,24}$ =	3.28	(NS, p=0.087)
Day 1	$F_{1,24}$ =	1.39	(NS, p=0.253)
Day 2	$F_{1,24}$ =	1.50	(NS, p=0.940)
Day 3	$F_{1,24}$ =	0.03	(NS, p=0.858)
Day 4	$F_{1,24}$ =	1.14	(NS, p=0.300)
Day 5	$F_{1,24}$ =	0.46	(NS, p=0.507)
Day 6	$F_{1,24}$ =	3.08	(NS, p=0.096)
Day 7	$F_{1,24}$ =	1.85	(NS, p=0.191)
Day 8	$F_{1,24}$ =	3.09	(NS, p=0.096)
Day 9	$F_{1,24}$ =	7.24	(p=0.015)
Day 10	$F_{1,24}$ =	5.85	(p=0.026)
Day 11	$F_{1,24}$ =	4.16	(NS, p=0.056)
Day 12	$F_{1,24}$ =	2.88	(NS, p=0.107)
Day 13	$F_{1,24}$ =	0.91	(NS, p=0.352)
Day 14	$F_{1,24}$ =	0.19	(NS, p=0.671)
Day 15	$F_{1,24}$ =	0.14	(NS, p=0.710)
Day 16	$F_{1,24}$ =	<0.01	(NS, p=0.949)
Day 17	$F_{1,24}$ =	<0.01	(NS, p=0.931)
Day 18	$F_{1,24}$ =	<0.01	(NS, p=0.943)
Day 19	$F_{1,24}$ =	0.21	(NS, p=0.655)
Day 20	$F_{1,24}$ =	<0.01	(NS, p=0.986)
Day 21	$F_{1,24}$ =	0.33	(NS, p=0.550)
Day 22	$F_{1,24}$ =	0.18	(NS, p=0.674)
Day 23	$F_{1,24}$ =	0.02	(NS, p=0.891)
Day 24	$F_{1,24}$ =	0.05	(NS, p=0.825)
Day 25	$F_{1,24}$ =	0.02	(NS, p=0.886)
Day 26	$F_{1,24}$ =	0.03	(NS, p=0.855)
Day 27	$F_{1,24}$ =	0.02	(NS, p=0.899)
Day 28	$F_{1,24}$ =	0.04	(NS, p=0.844)
Day 29	$F_{1,24}$ =	<0.01	(NS, p=0.946)
Day 30	$F_{1,24}$ =	<0.01	(NS, p=0.945)
Day 31	$F_{1,24}$ =	0.08	(NS, p=0.786)
Day 32	$F_{1,24}$ =	<0.01	(NS, p=0.979)
Day 33	$F_{1,24}$ =	0.02	(NS, p=0.886)
Day 34	$F_{1,24}$ =	0.07	(NS, p=0.789)
Day 35	$F_{1,24}$ =	0.10	(NS, p=0.754)
Day 36	$F_{1,24}$ =	0.05	(NS, p=0.819)
Day 37	$F_{1,24}$ =	0.01	(NS, p=0.918)
Day 38	$F_{1,24}$ =	<0.01	(NS, p=0.934)
Day 39	$F_{1,24}$ =	<0.01	(NS, p=0.966)
Day 40	$F_{1,24}$ =	0.06	(NS, p=0.807)
Day 41	$F_{1,24}$ =	0.05	(NS, p=0.831)
Day 42	$F_{1,24}$ =	0.12	(NS, p=0.731)
Day 43	$F_{1,24}$ =	0.36	(NS, p=0.557)
Day 44	$F_{1,24}$ =	0.84	(NS, p=0.373)
Day 45	$F_{1,24}$ =	0.59	(NS, p=0.453)
Day 46	$F_{1,24}$ =	0.36	(NS, p=0.555)
Day 47	$F_{1,24}$ =	0.64	(NS, p=0.434)
Day 48	$F_{1,24}$ =	0.76	(NS, p=0.396)
Day 49	$F_{1,24}$ =	0.70	(NS, p=0.413)
Day 50	$F_{1,24}$ =	0.52	(NS, p=0.478)
Day 51	$F_{1,24}$ =	0.37	(NS, p=0.549)

Figure 3.4. and 3.5. Food intake profile to day 51. Pages 103 and 105.

Adrenalectomy verses sham			D-Fen verses saline		
Day −3	$F_{1,24}$ =	2.40 (NS, p=0.139)	Day −3	$F_{1,24}$ =	1.09 (NS, p=0.309)
Day −2	$F_{1,24}$ =	3.38 (NS, p=0.083)	Day −2	$F_{1,24}$ =	0.17 (NS, p=0.688)
Day −1	$F_{1,24}$ =	1.94 (NS, p=0.181)	Day −1	$F_{1,24}$ =	0.20 (NS, p=0.662)
Day 0	$F_{1,24}$ =	1.13 (NS, p=0.302)	Day 0	$F_{1,24}$ =	<0.01 (NS, p=0.974)
Day 1	$F_{1,24}$ =	2.22 (NS, p=0.153)	Day 1	$F_{1,24}$ =	222.60 (p<0.001)
Day 2	$F_{1,24}$ =	0.38 (NS, p=0.546)	Day 2	$F_{1,24}$ =	23.15 (p<0.001)
Day 3	$F_{1,24}$ =	178.13 (p<0.001)	Day 3	$F_{1,24}$ =	30.18 (p<0.001)
Day 4	$F_{1,24}$ =	19.29 (p<0.001)	Day 4	$F_{1,24}$ =	<0.01 (NS, p=0.958)
Day 5	$F_{1,24}$ =	26.81 (p<0.001)	Day 5	$F_{1,24}$ =	7.42 (p=0.014)
Day 6	$F_{1,24}$ =	49.53 (p<0.001)	Day 6	$F_{1,24}$ =	1.77 (NS, p=0.200)
Day 7	$F_{1,24}$ =	14.14 (p=0.001)	Day 7	$F_{1,24}$ =	0.60 (NS, p=0.450)
Day 8	$F_{1,24}$ =	10.08 (p=0.005)	Day 8	$F_{1,24}$ =	5.22 (p=0.035)
Day 9	$F_{1,24}$ =	17.14 (p=0.001)	Day 9	$F_{1,24}$ =	10.31 (p=0.005)
Day 10	$F_{1,24}$ =	13.66 (p=0.002)	Day 10	$F_{1,24}$ =	0.04 (NS, p=0.836)
Day 11	$F_{1,24}$ =	0.88 (NS, p=0.362)	Day 11	$F_{1,24}$ =	0.80 (NS, p=0.384)
Day 12	$F_{1,24}$ =	2.30 (NS, p=0.147)	Day 12	$F_{1,24}$ =	9.27 (p=0.007)
Day 13	$F_{1,24}$ =	0.13 (NS, p=0.719)	Day 13	$F_{1,24}$ =	4.59 (p=0.046)
Day 14	$F_{1,24}$ =	<0.01 (NS, 0.946)	Day 14	$F_{1,24}$ =	1.06 (NS, p=0.318)
Day 15	$F_{1,24}$ =	<0.01 (NS, 0.946)	Day 15	$F_{1,24}$ =	1.06 (NS, p=0.318)
Day 16	$F_{1,24}$ =	0.67 (NS, 0.425)	Day 16	$F_{1,24}$ =	2.84 (NS, p=0.109)
Day 17	$F_{1,24}$ =	14.04 (p=0.001)	Day 17	$F_{1,24}$ =	0.13 (NS, p=0.726)
Day 18	$F_{1,24}$ =	3.63 (NS, p=0.073)	Day 18	$F_{1,24}$ =	0.04 (NS, p=0.850)
Day 19	$F_{1,24}$ =	2.30 (NS, p=0.147)	Day 19	$F_{1,24}$ =	0.14 (NS, p=0.710)
Day 20	$F_{1,24}$ =	5.91 (p=0.026)	Day 20	$F_{1,24}$ =	1.71 (NS, p=0.207)
Day 21	$F_{1,24}$ =	0.29 (NS, p=0.597)	Day 21	$F_{1,24}$ =	0.20 (NS, p=0.660)
Day 22	$F_{1,24}$ =	2.76 (NS, p=0.114)	Day 22	$F_{1,24}$ =	0.76 (NS, p=0.395)
Day 23	$F_{1,24}$ =	8.40 (p=0.010)	Day 23	$F_{1,24}$ =	7.05 (p=0.016)
Day 24	$F_{1,24}$ =	0.31 (NS, p=0.587)	Day 24	$F_{1,24}$ =	0.56 (NS, p=0.465)
Day 25	$F_{1,24}$ =	0.25 (NS, p=0.623)	Day 25	$F_{1,24}$ =	6.04 (p=0.024)
Day 26	$F_{1,24}$ =	0.57 (NS, p=0.459)	Day 26	$F_{1,24}$ =	0.08 (NS, p=0.784)
Day 27	$F_{1,24}$ =	0.57 (NS, p=0.459)	Day 27	$F_{1,24}$ =	0.31 (NS, p=0.584)
Day 28	$F_{1,24}$ =	0.96 (NS, p=0.339)	Day 28	$F_{1,24}$ =	5.08 (p=0.037)
Day 29	$F_{1,24}$ =	3.21 (NS, p=0.090)	Day 29	$F_{1,24}$ =	2.36 (NS, p=0.142)
Day 30	$F_{1,24}$ =	0.92 (NS, p=0.627)	Day 30	$F_{1,24}$ =	0.03 (NS, p=0.856)
Day 31	$F_{1,24}$ =	0.92 (NS, p=0.351)	Day 31	$F_{1,24}$ =	0.02 (NS, p=0.878)
Day 32	$F_{1,24}$ =	0.19 (NS, p=0.670)	Day 32	$F_{1,24}$ =	0.06 (NS, p=0.817)
Day 33	$F_{1,24}$ =	0.21 (NS, p=0.655)	Day 33	$F_{1,24}$ =	0.11 (NS, p=0.747)
Day 34	$F_{1,24}$ =	<0.01 (NS, p=0.955)	Day 34	$F_{1,24}$ =	1.40 (NS, p=0.251)
Day 35	$F_{1,24}$ =	2.18 (NS, p=0.158)	Day 35	$F_{1,24}$ =	4.07 (NS, p=0.059)
Day 36	$F_{1,24}$ =	0.53 (NS, p=0.478)	Day 36	$F_{1,24}$ =	0.07 (NS, p=0.795)
Day 37	$F_{1,24}$ =	0.03 (NS, p=0.863)	Day 37	$F_{1,24}$ =	<0.01 (NS, p=0.954)
Day 38	$F_{1,24}$ =	0.97 (NS, p=0.337)	Day 38	$F_{1,24}$ =	7.42 (p=0.014)
Day 39	$F_{1,24}$ =	0.62 (NS, p=0.441)	Day 39	$F_{1,24}$ =	0.41 (NS, p=0.530)
Day 40	$F_{1,24}$ =	2.16 (NS, p=0.159)	Day 40	$F_{1,24}$ =	0.02 (NS, p=0.967)
Day 41	$F_{1,24}$ =	2.23 (NS, p=0.153)	Day 41	$F_{1,24}$ =	0.85 (NS, p=0.370)
Day 42	$F_{1,24}$ =	3.28 (NS, p=0.087)	Day 42	$F_{1,24}$ =	4.95 (p=0.039)
Day 43	$F_{1,24}$ =	<0.01 (NS, p=0.987)	Day 43	$F_{1,24}$ =	0.45 (NS, p=0.511)
Day 44	$F_{1,24}$ =	2.52 (NS, p=0.130)	Day 44	$F_{1,24}$ =	1.73 (NS, p=0.204)
Day 45	$F_{1,24}$ =	0.04 (NS, p=0.841)	Day 45	$F_{1,24}$ =	0.47 (NS, p=0.500)
Day 46	$F_{1,24}$ =	0.46 (NS, p=0.505)	Day 46	$F_{1,24}$ =	6.77 (p=0.018)
Day 47	$F_{1,24}$ =	3.76 (NS, p=0.068)	Day 47	$F_{1,24}$ =	0.12 (NS, p=0.731)
Day 48	$F_{1,24}$ =	0.15 (NS, p=0.706)	Day 48	$F_{1,24}$ =	0.29 (NS, p=0.598)
Day 49	$F_{1,24}$ =	1.29 (NS, p=0.271)	Day 49	$F_{1,24}$ =	0.22 (NS, p=0.646)
Day 50	$F_{1,24}$ =	2.24 (NS, p=0.152)	Day 50	$F_{1,24}$ =	7.32 (p=0.015)

Figure 3.4. and 3.5. Food intake profile to day 51. Pages 103 and 105 (continued).

Interaction

Day –3	$F_{1,24}$ =	3.92	(NS, p=0.063)
Day –2	$F_{1,24}$ =	3.25	(NS, p=0.088)
Day –1	$F_{1,24}$ =	3.43	(NS, p=0.081)
Day 0	$F_{1,24}$ =	0.94	(NS, p=0.345)
Day 1	$F_{1,24}$ =	0.46	(NS, p=0.504)
Day 2	$F_{1,24}$ =	0.30	(NS, p=0.588)
Day 3	$F_{1,24}$ =	0.27	(NS, p=0.612)
Day 4	$F_{1,24}$ =	2.18	(NS, p=0.157)
Day 5	$F_{1,24}$ =	0.10	(NS, p=0.760)
Day 6	$F_{1,24}$ =	2.33	(NS, p=0.144)
Day 7	$F_{1,24}$ =	6.80	(p=0.018)
Day 8	$F_{1,24}$ =	1.80	(NS, p=0.197)
Day 9	$F_{1,24}$ =	0.18	(NS, p=0.675)
Day 10	$F_{1,24}$ =	0.81	(NS, p=0.379)
Day 11	$F_{1,24}$ =	11.81	(p=0.003)
Day 12	$F_{1,24}$ =	4.97	(p=0.039)
Day 13	$F_{1,24}$ =	1.34	(NS, p=0.263)
Day 14	$F_{1,24}$ =	0.51	(NS, p=0.483)
Day 15	$F_{1,24}$ =	0.51	(NS, p=0.483)
Day 16	$F_{1,24}$ =	1.18	(NS, p=0.292)
Day 17	$F_{1,24}$ =	0.01	(NS, p=0.911)
Day 18	$F_{1,24}$ =	0.14	(NS, p=0.709)
Day 19	$F_{1,24}$ =	<0.01	(NS, p=0.978)
Day 20	$F_{1,24}$ =	1.83	(NS, p=0.193)
Day 21	$F_{1,24}$ =	0.10	(NS, p=0.753)
Day 22	$F_{1,24}$ =	0.33	(NS, p=0.574)
Day 23	$F_{1,24}$ =	1.63	(NS, p=0.218)
Day 24	$F_{1,24}$ =	0.50	(NS, p=0.487)
Day 25	$F_{1,24}$ =	0.53	(NS, p=0.477)
Day 26	$F_{1,24}$ =	0.11	(NS, p=0.749)
Day 27	$F_{1,24}$ =	2.03	(NS, p=0.172)
Day 28	$F_{1,24}$ =	0.04	(NS, p=0.850)
Day 29	$F_{1,24}$ =	0.73	(NS, p=0.404)
Day 30	$F_{1,24}$ =	4.86	(p=0.041)
Day 31	$F_{1,24}$ =	0.32	(NS, p=0.578)
Day 32	$F_{1,24}$ =	3.99	(NS, p=0.061)
Day 33	$F_{1,24}$ =	3.89	(NS, p=0.064)
Day 34	$F_{1,24}$ =	2.10	(NS, p=0.164)
Day 35	$F_{1,24}$ =	0.05	(NS, p=0.829)
Day 36	$F_{1,24}$ =	0.40	(NS, p=0.535)
Day 37	$F_{1,24}$ =	2.62	(NS, p=0.123)
Day 38	$F_{1,24}$ =	0.05	(NS, p=0.834)
Day 39	$F_{1,24}$ =	0.63	(NS, p=0.439)
Day 40	$F_{1,24}$ =	0.44	(NS, p=0.517)
Day 41	$F_{1,24}$ =	0.08	(NS, p=0.786)
Day 42	$F_{1,24}$ =	0.93	(NS, p=0.621)
Day 43	$F_{1,24}$ =	6.48	(p=0.020)
Day 44	$F_{1,24}$ =	2.73	(NS, p=0.116)
Day 45	$F_{1,24}$ =	0.01	(NS, p=0.911)
Day 46	$F_{1,24}$ =	0.48	(NS, p=0.495)
Day 47	$F_{1,24}$ =	0.66	(NS, p=0.427)
Day 48	$F_{1,24}$ =	0.59	(NS, p=0.453)
Day 49	$F_{1,24}$ =	0.02	(NS, p=0.903)
Day 50	$F_{1,24}$ =	4.52	(p=0.048)

Figure 3.7. Body weight profile to day 8. Page 135.

Adrenalectomy verses sham			D-Fen verses saline		
Day −2	$F_{1,20}$ =	0.02 (NS, p=0.884)	Day −2	$F_{1,20}$ =	0.02 (NS, p=0.884)
Day −1	$F_{1,20}$ =	1.58 (NS, p=0.227)	Day −1	$F_{1,20}$ =	1.58 (NS, p=0.227)
Day 0	$F_{1,20}$ =	3.52 (NS, p=0.079)	Day 0	$F_{1,20}$ =	2.78 (NS, p=0.115)
Day 1	$F_{1,20}$ =	9.11 (p=0.08)	Day 1	$F_{1,20}$ =	2.74 (NS, p=0.117)
Day 2	$F_{1,20}$ =	18.05 (p=0.128)	Day 2	$F_{1,20}$ =	30.18 (p<0.001)
Day 3	$F_{1,20}$ =	4.99 (p=0.004)	Day 3	$F_{1,20}$ =	34.05 (p<0.001)
Day 4	$F_{1,20}$ =	9.65 (p=0.007)	Day 4	$F_{1,20}$ =	23.82 (p<0.001)
Day 5	$F_{1,20}$ =	25.32 (p<0.001)	Day 5	$F_{1,20}$ =	27.28 (p<0.001)
Day 6	$F_{1,20}$ =	36.71 (p<0.001)	Day 6	$F_{1,20}$ =	44.03 (p<0.001)
Day 7	$F_{1,20}$ =	53.12 (p<0.001)	Day 7	$F_{1,20}$ =	93.76 (p<0.001)
Day 8	$F_{1,20}$ =	48.07 (p<0.001)	Day 8	$F_{1,20}$ =	75.85 (p<0.001)
Interaction					
Day −2	$F_{1,20}$ =	0.20 (NS, p=0.662)			
Day −1	$F_{1,20}$ =	0.33 (NS, p=0.575)			
Day 0	$F_{1,20}$ =	0.04 (NS, p=0.837)			
Day 1	$F_{1,20}$ =	1.15 (NS, p=0.300)			
Day 2	$F_{1,20}$ =	1.21 (NS, p=0.288)			
Day 3	$F_{1,20}$ =	<0.01 (NS, p=1.000)			
Day 4	$F_{1,20}$ =	10.27 (p=0.006)			
Day 5	$F_{1,20}$ =	7.99 (p=0.012)			
Day 6	$F_{1,20}$ =	4.08 (NS, p=0.061)			
Day 7	$F_{1,20}$ =	8.50 (p=0.010)			
Day 8	$F_{1,20}$ =	11.44 (p=0.004)			

Figure 3.8. Food intake profile to day 8. Page 138.

Adrenalectomy verses sham			D-Fen verses saline		
Day −3	$F_{1,20}$ =	10.07 (p=0.006)	Day −3	$F_{1,20}$ =	4.11 (NS, p=0.060)
Day −2	$F_{1,20}$ =	1.89 (NS, p=0.189)	Day −2	$F_{1,20}$ =	0.86 (NS, p=0.368)
Day −1	$F_{1,20}$ =	4.18 (NS, p=0.058)	Day −1	$F_{1,20}$ =	1.75 (NS, p=0.205)
Day 0	$F_{1,20}$ =	0.23 (NS, p=0.636)	Day 0	$F_{1,20}$ =	0.52 (NS, p=0.479)
Day 1	$F_{1,20}$ =	0.01 (NS, p=0.928)	Day 1	$F_{1,20}$ =	175.53 (p<0.001)
Day 2	$F_{1,20}$ =	15.33 (p=0.001)	Day 2	$F_{1,20}$ =	37.45 (p<0.001)
Day 3	$F_{1,20}$ =	760.17 (p<0.001)	Day 3	$F_{1,20}$ =	973.50 (p<0.001)
Day 4	$F_{1,20}$ =	4.58 (p=0.048)	Day 4	$F_{1,20}$ =	15.65 (p=0.001)
Day 5	$F_{1,20}$ =	18.93 (p<0.001)	Day 5	$F_{1,20}$ =	14.89 (p=0.001)
Day 6	$F_{1,20}$ =	26.78 (p<0.001)	Day 6	$F_{1,20}$ =	9.23 (p=0.008)
Day 7	$F_{1,20}$ =	17.05 (p=0.001)	Day 7	$F_{1,20}$ =	10.14 (p=0.006)
Interaction					
Day −2	$F_{1,20}$ =	2.70 (NS, p=0.120)			
Day −1	$F_{1,20}$ =	0.69 (NS, p=0.418)			
Day 0	$F_{1,20}$ =	0.11 (NS, p=0.748)			
Day 1	$F_{1,20}$ =	0.33 (NS, p=0.574)			
Day 2	$F_{1,20}$ =	1.14 (NS, p=0.303)			
Day 3	$F_{1,20}$ =	13.50 (p=0.002)			
Day 4	$F_{1,20}$ =	9.08 (p=0.008)			
Day 5	$F_{1,20}$ =	0.33 (NS, p=0.574)			
Day 6	$F_{1,20}$ =	0.41 (NS, p=0.529)			
Day 7	$F_{1,20}$ =	2.07 (NS, p=0.169)			

APPENDIX 2. (TABLES 2 way ANOVA F / p values)

Table 3.1. Glucose, insulin, liver fat mass for day 7 and 51. Page 108.

1.	$F_{1,24}$ =	11.14	(p=0.003)
2.	$F_{1,24}$ =	1.99	(NS, p=0.174)
3.	$F_{1,24}$ =	0.67	(NS, p=0.422)
4.	$F_{1,24}$ =	4.66	(p=0.046)
5.	$F_{1,24}$ =	3.54	(NS, p=0.077)
6.	$F_{1,24}$ =	0.97	(NS, p=0.339)
7.	$F_{1,24}$ =	21.57	(p<0.001)
8.	$F_{1,24}$ =	0.16	(NS, p=0.696)
9.	$F_{1,24}$ =	1.29	(NS, p=0.269)
10.	$F_{1,24}$ =	9.15	(p=0.008)
11.	$F_{1,24}$ =	0.07	(NS, p=0.794)
12.	$F_{1,24}$ =	0.87	(NS, p=0.364)
13.	$F_{1,24}$ =	14.74	(p=0.001)
14.	$F_{1,24}$ =	11.61	(p=0.003)
15.	$F_{1,24}$ =	0.27	(NS, p=0.607)
16.	$F_{1,24}$ =	5.56	(p=0.030)
17.	$F_{1,24}$ =	0.25	(NS, p=0.623)
18.	$F_{1,24}$ =	0.40	(NS, p=0.538)
19.	$F_{1,24}$ =	34.03	(p<0.001)
20.	$F_{1,24}$ =	5.99	(p=0.024)
21.	$F_{1,24}$ =	0.09	(NS, p=0.768)
22.	$F_{1,24}$ =	34.85	(p=0.001)
23.	$F_{1,24}$ =	0.98	(NS, p=0.336)
24.	$F_{1,24}$ =	1.38	(NS, p=0.256)

Table 3.2. Total food intake and body weight change for day 7. Page 111.

1.	$F_{1,24}$ =	243.66	(p<0.001)
2.	$F_{1,24}$ =	318.93	(p<0.001)
3.	$F_{1,24}$ =	6.95	(p=0.016)
4.	$F_{1,24}$ =	30.55	(p<0.001)
5.	$F_{1,24}$ =	28.27	(p<0.001)
6.	$F_{1,24}$ =	1.96	(NS, p=0.177)

Table 3.3. Total food intake and body weight change for day 51. Page 111.

1.	$F_{1,24}$ =	65.33	(p<0.001)
2.	$F_{1,24}$ =	55.22	(p<0.001)
3.	$F_{1,24}$ =	4.85	(p=0.041)
4.	$F_{1,24}$ =	6.70	(p<0.019)
5.	$F_{1,24}$ =	2.99	(NS, p=0.101)
6.	$F_{1,24}$ =	0.09	(NS, p=0.763)
7.	$F_{1,24}$ =	9.66	(p=0.009)
8.	$F_{1,24}$ =	8.61	(p=0.013)
9.	$F_{1,24}$ =	6.70	(p<0.011)
10.	$F_{1,24}$ =	3.60	(NS, p=0.07)
11.	$F_{1,24}$ =	20.22	(p<0.001)
12.	$F_{1,24}$ =	1.14	(NS, p=0.300)

Table 3.4. Hypothalamic 5-HT, 5-HIAA, Ratio and Tryptophan day 7 and 51. Page 118.

1.	$F_{1,24}$ =	9.39	(p=0.007)
2.	$F_{1,24}$ =	4.80	(p=0.021)
3.	$F_{1,24}$ =	0.60	(NS, p=0.450)
4.	$F_{1,24}$ =	0.02	(NS, p=0.901)
5.	$F_{1,24}$ =	12.07	(p=0.003)
6.	$F_{1,24}$ =	0.01	(NS, p=0.916)
7.	$F_{1,24}$ =	<0.01	(NS, p=0.956)
8.	$F_{1,24}$ =	7.67	(p=0.013)
9.	$F_{1,24}$ =	0.35	(NS, p=0.561)
10.	$F_{1,24}$ =	0.31	(NS, p=0.588)
11.	$F_{1,24}$ =	8.96	(p=0.009)
12.	$F_{1,24}$ =	<0.01	(NS, p=0.944)
13.	$F_{1,24}$ =	11.53	(p=0.003)
14.	$F_{1,24}$ =	10.87	(p=0.004)
15.	$F_{1,24}$ =	2.54	(NS, p=0.129)
16.	$F_{1,24}$ =	0.24	(NS, p=0.629)
17.	$F_{1,24}$ =	0.24	(NS, p=0.631)
18.	$F_{1,24}$ =	0.03	(NS, p=0.876)
19.	$F_{1,24}$ =	<0.01	(NS, p=0.997)
20.	$F_{1,24}$ =	0.49	(NS, p=0.494)
21.	$F_{1,24}$ =	0.03	(NS, p=0.867)
22.	$F_{1,24}$ =	0.05	(NS, p=0.819)
23.	$F_{1,24}$ =	2.38	(NS, p=0.144)
24.	$F_{1,24}$ =	2.37	(NS, p=0.145)

Table 3.5. Hippocampal 5-HT, 5-HIAA, Ratio and Tryptophan day 7 and 51. Page 121.

1.	$F_{1,24}$ =	1.71	(NS, p=0.207)
2.	$F_{1,24}$ =	19.62	(p<0.001)
3.	$F_{1,24}$ =	0.01	(NS, p=0.935)
4.	$F_{1,24}$ =	1.77	(NS, p=0.203)
5.	$F_{1,24}$ =	32.62	(p<0.001)
6.	$F_{1,24}$ =	0.14	(NS, p=0.713)
7.	$F_{1,24}$ =	1.02	(NS, p=0.326)
8.	$F_{1,24}$ =	14.86	(p=0.001)
9.	$F_{1,24}$ =	0.05	(NS, p=0.824)
10.	$F_{1,24}$ =	5.84	(p=0.029)
11.	$F_{1,24}$ =	29.98	(p<0.001)
12.	$F_{1,24}$ =	0.01	(NS, p=0.925)
13.	$F_{1,24}$ =	0.63	(NS, p=0.438)
14.	$F_{1,24}$ =	0.71	(NS, p=0.409)
15.	$F_{1,24}$ =	0.05	(NS, p=0.820)
16.	$F_{1,24}$ =	0.01	(NS, p=0.928)
17.	$F_{1,24}$ =	29.47	(p<0.001)
18.	$F_{1,24}$ =	1.15	(NS, p=0.301)
19.	$F_{1,24}$ =	5.67	(p=0.028)
20.	$F_{1,24}$ =	0.78	(NS, p=0.389)
21.	$F_{1,24}$ =	3.26	(NS, p=0.087)
22.	$F_{1,24}$ =	1.55	(NS, p=0.232)
23.	$F_{1,24}$ =	0.76	(NS, p=0.397)
24.	$F_{1,24}$ =	0.10	(NS, p=0.758)

Table 3.6. Body composition analysis day 7 and 51. Page 126.

1.	$F_{1,24}$ =	9.28	(p=0.006)
2.	$F_{1,24}$ =	6.60	(p=0.018)
3.	$F_{1,24}$ =	1.78	(NS, p=0.197)
4.	$F_{1,24}$ =	0.10	(NS, p=0.757)
5.	$F_{1,24}$ =	0.31	(NS, p=0.589)
6.	$F_{1,24}$ =	1.01	(NS, p=0.330)
7.	$F_{1,24}$ =	10.49	(p=0.004)
8.	$F_{1,24}$ =	0.57	(NS, p=0.459)
9.	$F_{1,24}$ =	1.64	(NS, p=0.214)
10.	$F_{1,24}$ =	28.49	(p<0.001)
11.	$F_{1,24}$ =	1.09	(NS, p=0.313)
12.	$F_{1,24}$ =	2.04	(NS, p=0.172)
13.	$F_{1,24}$ =	27.01	(p<0.001)
14.	$F_{1,24}$ =	2.11	(NS, p=0.162)
15.	$F_{1,24}$ =	3.02	(NS, p=0.098)
16.	$F_{1,24}$ =	16.74	(p<0.001)
17.	$F_{1,24}$ =	0.32	(NS, p=0.580)
18.	$F_{1,24}$ =	2.06	(NS, p=0.170)

Table 3.7. Glucose, insulin, liver fat mass for day 8. Page 141.

1.	$F_{1,20}$ =	12.98	(p=0.002)
2.	$F_{1,20}$ =	<0.01	(NS, p=0.951)
3.	$F_{1,20}$ =	2.12	(NS, p=0.164)
4.	$F_{1,20}$ =	2.52	(NS, p=0.132)
5.	$F_{1,20}$ =	0.13	(NS, p=0.719)
6.	$F_{1,20}$ =	<0.01	(NS, p=0.965)
7.	$F_{1,20}$ =	18.43	(p=0.001)
8.	$F_{1,20}$ =	9.14	(p=0.008)
9.	$F_{1,20}$ =	1.12	(NS, p=0.307)
10.	$F_{1,20}$ =	24.51	(p<0.001)
11.	$F_{1,20}$ =	8.82	(p=0.009)
12.	$F_{1,20}$ =	1.53	(NS, p=0.234)

Table 3.8. Total food intake and body weight change for day 8. Page 143.

1.	$F_{1,20}$ =	104.83	(p<0.001)
2.	$F_{1,20}$ =	134.51	(p<0.001)
3.	$F_{1,20}$ =	6.97	(p=0.018)
4.	$F_{1,20}$ =	61.32	(p<0.001)
5.	$F_{1,20}$ =	136.04	(p<0.001)
6.	$F_{1,20}$ =	19.67	(p<0.001)

Table 3.9. Hypothalamic 5-HT, 5-HIAA, Ratio and Tryptophan day 8. Page 146.

1.	$F_{1,20}$ =	4.01	(NS, p=0.064)
2.	$F_{1,20}$ =	46.78	(p<0.001)
3.	$F_{1,20}$ =	<0.01	(NS, p=0.971)
4.	$F_{1,20}$ =	0.07	(NS, p=0.798)
5.	$F_{1,20}$ =	43.00	(p<0.001)
6.	$F_{1,20}$ =	<0.01	(NS, p=0.925)
7.	$F_{1,20}$ =	6.00	(p=0.027)
8.	$F_{1,20}$ =	19.20	(p=0.001)
9.	$F_{1,20}$ =	1.20	(NS, p=0.290)
10.	$F_{1,20}$ =	1.03	(NS, p=0.327)
11.	$F_{1,20}$ =	0.21	(NS, p=0.651)
12.	$F_{1,20}$ =	2.55	(NS, p=0.131)

Table 3.10. Hippocampal 5-HT, 5-HIAA, Ratio and Tryptophan day 8. Page 149.

1.	$F_{1,20}$ =	0.57	(NS, p=0.461)
2.	$F_{1,20}$ =	36.37	(p<0.001)
3.	$F_{1,20}$ =	4.69	(p=0.048)
4.	$F_{1,20}$ =	0.08	(NS, p=0.780)
5.	$F_{1,20}$ =	105.89	(p<0.001)
6.	$F_{1,20}$ =	0.22	(NS, p=0.645)
7.	$F_{1,20}$ =	1.58	(NS, p=0.229)
8.	$F_{1,20}$ =	0.24	(NS, p=0.635)
9.	$F_{1,20}$ =	0.16	(NS, p=0.692)
10.	$F_{1,20}$ =	4.42	(NS, p=0.054)
11.	$F_{1,20}$ =	1.31	(NS, p=0.272)
12.	$F_{1,20}$ =	6.32	(p=0.025)