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ENERGY HOMEOSTASIS:

CROSSTALK BETWEEN ADIPOSE TISSUE AND THE HUMAN HYPOTHALAMUS

By

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A thesis submitted to
the Faculty of Medicine
of the University of Warwick
for the degree of

Doctor of Philosophy

July 2007

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Cheshire, autumn 2006

One need not be a rocket scientist to notice that increased food intake tends to be associated with obesity (Spiegelman, 1996); however, to understand the regulation of energy homeostasis in all its complexity will require boldly go where no molecular biologist has gone before.

ACKNOWLEDGEMENTS

Many thanks to my supervisors Professor Sudhesh Kumar, Philip McTernan and Paul O'Hare for making this thesis possible and for their guidance on scientific matters. Thanks to Paul O'Hare specifically, with his creativity in obtaining funding for my research. This included exposure to the world of educationalists with an experience as GLF (group learning facilitator) at Warwick Medical School for me.

Thanks to the anaesthetists and surgeons at Sollihull Hospital. Particular thanks go to all the patients who consented to donate samples for these studies and so made this research possible. Thanks to Sean James for his help with Immunohistochemistry and Anton Tonchev from Varna University, Bulgaria for sharing some of his hypothalamic human samples. Thanks to Adam Baker who helped with the microarray analysis and the jolly company of other PhD students in the lab. Thanks to all the secretaries and all helpers behind the scenes who helped me to keep high spirits and get going when the going got hard.

Great thanks to my whole family, especially my husband Pierre (my star and star scientist) for his patience and for coping with separation for almost three years since the workplace of my thesis left us 70 miles apart. Thanks very much to my mother–in-law, Nicole Maxted, for the proofreading at short notice and her contribution of numerous commas.

Finally, thanks to my cat (Posh) for calming my nerves. She suffered from being ignored and envied the laptop as the preferred thing on my lap instead of her. Doing what cats do, she bit the tail of the mouse off (the computer mouse) which resulted in prompt shortcut of the laptop systems but no change of her fortune.

DECLARATION

I declare that this thesis is a record of results obtained by me based on my own laboratory work, with the exception of the immunohistochemical analysis, where I received help by Sean James, and the microarray analysis by Adam Baker. This thesis was written by me and lead to publications as result of the work. The results of chapter 3 are about to be published in the Journal of Endocrinology and Metabolism. Co-authors aided in supervision, help with immunohistology and supply of human brain tissue. I have obtained the ethics for the attached paper of Kusminski *et. al.* (2007) and contributed with patient recruitment, dealt with the sampling, insulin and glucose analysis and contributed with intellectual input.

None of the work has been previously submitted for a higher degree.
All sources have been specifically acknowledged by means of reference.

SYNOPSIS

There is a worldwide epidemic of obesity. Weight rise is a consequence of continuous positive energy balance which leads to accumulation of body fat. Recent insights into adipose tissue (AT) biology have led to the conclusion that the adipocyte is not just a storage depot for triglycerides but also an endocrine organ. AT secretes proteins, such as leptin, which control central appetite regulation in the human hypothalamus. In contrast, several other proteins and neurotransmitters regulate central energy balance, but can also influence AT metabolism to elicit feedback on fat accumulation. This suggests a close link between AT and the brain within an AT-to-brain crosstalk system including feedback circuits. This thesis examines firstly, the potential of crosstalk between AT and the brain by other adipokines and secondly, the brain-AT crosstalk by expression of neurotransmitters and their receptors in AT. The study establishes the presence of the adipokines adiponectin and resistin in human cerebrospinal fluid and immunohistochemistry showed adiponectin receptors in energy regulating nuclei of the hypothalamus. Furthermore, this thesis established that the orexigenic neurotransmitters NPY and ghrelin are secreted by human adipocytes, where they enhance lipid accumulation. Further, that NPY levels increase with obesity and its *in vitro* secretion is enhanced by insulin. This may play an important role in the pathogenesis of the metabolic syndrome and may induce an escape of the appetite behaviour towards positive energy balance. Finally, this thesis highlights the influence of a depot-specific innervation of AT on energy homeostasis by establishing presence of nicotinic receptors in human adipocytes, which may play a role in smoking induced changes in adipokine secretion and fat mass.

In conclusion, this thesis suggests a tight interplay between AT and the brain and highlights its potential relevance in human pathophysiology.

ABBREVIATIONS

Abd	abdominal
AdipoR	adiponectin receptor
AMPK	adenosine monophosphate kinase
ACTH	adenocorticotrophic hormone
AGRP	agouti related protein pathway
αMSH	α-melanin stimulating hormone
ARC	arcuate nucleus
ASP	adiopokine acetylation stimulating protein
AT	adipose tissue
BAT	brown adipose tissue
BBB	blood brain barrier
BMI	body mass index
BMR	basic metabolic rate
BSA	bovine serum albumin
cAMP	cyclic adenosine-3',5'-monophosphate
CART	cocaine-and amphetamine-regulated transcript
cDNA	complementary (to mRNA) deoxyribonucleic acid
c-Fos	cellular Fos nuclear protooncogene
CHRNA	cholinergic nicotinic receptor alpha
cm	centimetre
cRNA	complementary ribonucleic acid
CRP	c-reactive protein
CNS	central nervous system
CSF	cerebrospinal fluid
DMEM	Dulbecco's minimum essential medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPS	deoxynucleotides triphosphates
DPP-IV	dipeptidyl peptidase IV
DTT	dithiothreitol
ECL (+)	enhanced chemiluminescence (plus)
EDTA	ethylenediaminetetraacetic acid

FFA	free fatty acid
FIZZ	found in the inflammatory zone
FCS	foetal calf serum
g	gram
x g	factor of gravity
GLP-1	glucagon like peptide-1
GLUT	glucose transporter protein
GHS-R	growth hormone secretagogue receptor
HMW	Higher Molecular Weight
HOMA-IR	Homeostasis Assessment Model of Insulin Resistance
hr	hours
HSL	hormone sensitive lipase
IGT	impaired glucose tolerance
icv	intracerebroventrical
IL	Interleukin
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
JAK	Janus- family tyrosine kinase
kDa	kilodalton
L	litre
LMW	lower molecular weight
LPL	lipoprotein lipase
M	molar
MI	myocardial infarction
min	minute (time)
μL	microlitre
mL	millilitre
mM	millimolar
mQH_20	milli Q water (ultra filtered water)
mRNA	messenger ribonucleic acid
mg	milligram
μg	microgram
NA	noradrenaline

NEFA	non esterified fatty acids
NFκB	Nuclear Factor Kappa B
ng	nanogram
nmoL	nanomole
NPY	Neuropeptide Y
Y	Neuropeptide receptor
NS	non significant
NTS	nucleus tractus solitarius
OD	optical density
PAI	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with tween 20
PI3K	phosphatidylinositol 3-kinase
POMC	proopiomelanocortin
PPAR	Peroxisome Proliferated Activated Receptor
PVN	paraventricular nucleus
PYY	peptide YY
rh	recombinant
RNA	ribonucleic acid
RSG	rosiglitazone
RT	room temperature
s	second (time)
Sc	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SNS	sympathetic nervous system
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
T2DM	Type Two Diabetes Mellitus
Taq	*Thermus aquaticus* (DNA polymerase)
TEMED	N, N, N', N' -tetramethylethelenediamine
TG	triglyceride

Th	Thigh
TNFα	Tumour necrosis factor alpha
Tris	tris (hydroxymethyl) aminomethane
TZD	thiazolidinediones
u	units
UV	ultraviolet
WAT	white adipose tissue
11ß-HSD	11ß-hydroxysteroid-dehydrogenase

Chapter 1

Introduction

1.1. Obesity

Obesity is a condition in which excess fat has accumulated to such an extent that health is adversely affected (WHO, 1999) and is a consequence of continuous positive energy balance. This thesis explores the mechanisms of fat deposition and feedback mechanisms of energy homeostasis and in particular examines adipose tissue and its crosstalk with principle regulators of central energy balance.

1.1.1. Obesity: The epidemic

Worldwide, 300 million of the adult population are overweight or obese (WHO, 2003). The number of obese and overweight people in the UK has tripled over the last two decades and more than 50% of British people have been categorised as obese (National Audit Office 2001). Based on observations taken over 10 years, the Department of Health Annual Health Survey for England (2003) estimated a continuous average weight gain of about 0.35kg/year per adult person. Obesity has also begun to replace the more traditional causes of ill health like undernutrition and infectious diseases in third word countries (WHO, 2003). These epidemic proportions of obesity are not restricted to adults and there is an even bigger concern in children (Chinn, 2001). With growing rates of childhood obesity, obesity related diseases and T2DM are increasingly seen at younger ages (Kaufman, 2002). Obesity is a chronic, progressive and relapsing disease and its management is a challenge (Kos, 2004). A closer understanding of human energy regulation gives hope to the development of much required effective treatments.

1.1.2 Definition of obesity and the metabolic syndrome

The definition of obesity currently used by the WHO is based on the measurement of a person's body mass index (BMI). This is calculated by dividing the person's weight in kilograms by the square of their height in metres (kg/m^2). A BMI of >30 kg/m^2 is classified as obesity and the range between 25 to 30 kg/m^2 as overweight.

WHO classification for Europids	BMI
Underweight	<18.5
Healthy/Lean	18.5-24.9
Overweight	25-29.9
Obese I	30-34.9
Obese II	35-39.9
Morbid Obesity	>40

Table 1.1.2: Obesity classification (Weisell, 2002).

BMI is the most routinely used parameter to indirectly measure 'fatness', due to its ease of determination and calculation. However, the risk of obesity related type 2 diabetes (T2DM) is better defined by a high waist-hip ratio (WHR) (Larsson, 1984) as the BMI index does not identify extremes of those with high fat or high muscle mass. Since abdominal fat as reflected by waist circumference is more harmful to health than other fat depots and correlates better with cardiovascular risk (Dobbelsteyn, 2001), the recent trend includes the combination of waist circumference as well as BMI in epidemiological studies. The use of CT scan, DEXA and MRI allows the measurement of depot specific distribution of adipose tissue, e.g. with cross sectional views of the abdomen. Other tools of body fat estimation are fat measurements with skin

callipers, the use of bioelectrical impedance (radio-frequency pulse), water immersion test and the less invasive air-displacement plethysmography (BOD POD) (Fields, 2002). While these methods are useful in research, they are not suitable, or their use justifiable, for routine clinical risk stratification.

1.1.3 Obesity and adverse health

Obesity has adverse health effects and leads to premature death (Calle, 1999). Death is caused indirectly and thus an exact calculation of obesity related mortality is difficult. However, there are estimates of a decrease of life expectancy by 3 to 14 years (Jebb, 2003). The mortality increases sharply with BMI above 30 kg/m^2 (Williamson, 1995; Allison, 1999) and is positively related to the duration of obesity. Obesity also increases the risk of certain cancers, *e.g.* endometrial, colon and postmenopausal breast cancer (Calle, 2003) and is positively correlated with the occurrence of sleep apnoea, joint disease and depression. Most importantly obesity is correlated with hypertension, dyslipidaemia and progressive increase of insulin resistance which can result in T2DM. The combination of these disease entities is also known as the metabolic syndrome, or 'deadly quartet', and each individual component is associated with an increased incidence of cardiovascular disease and stroke. There is no unifying definition for the metabolic syndrome and no agreement by expert groups on the individual benchmarks of the clinical criteria for identification of this syndrome cluster (Alberti, 2005), which makes an estimate of its prevalence difficult. The role of the individual components in the pathogenesis of this syndrome is unclear and obesity and possibly insulin resistance are considered as their main drivers. The development of obesity related T2DM is a transient

process commencing with insulin resistance in which there is a compensatory increase in insulin production and pancreatic β-cells secretion (DeFronzo, 1988; Raeven, 1989). This systemic hyperinsulinaemia is initially able to suppress hepatic insulin production and maintain normal glucose levels. With increased strain of the β-cells the condition progresses to impaired glucose tolerance (IGT) and finally to overt T2DM which is defined by fasting hyperglycaemia (Bogardus, 1984; WHO, 1999). As the prevalence of obesity increases, the obesity epidemic is predicted to be followed by a diabetes epidemic (Mokdad, 2003) and the term 'diabesity' has been proposed with the increasing recognition of a connection between obesity and T2DM (Astrup, 2000). The association becomes apparent with the increased average BMI at diagnosis of T2DM, which is 28-29 kg/m^2 (UKPDS Group, 1998). Data from the Nurses Health Study and Health Professionals Follow up Study (Chan, 1994; Colditz, 1995) indicate that in subjects of a BMI of 35 kg/m^2, the relative risk of developing T2DM increases by 40 fold in women and by 60 fold in men. A link between obesity and T2DM is also supported by the effect of weight loss, which can prevent and delay the onset of T2DM (Tuomilheto, 2001), alongside other metabolic complications of obesity. Weight loss of 10% of body weight not only reduces the blood pressure and cholesterol, but also reduces the risk of diabetes onset by 50% and decreases the HbA1C by 15% (Jung, 1997). The exact mechanism of obesity related T2DM is yet poorly understood and most likely multifactorial.

1.1.4. Obesity as dysregulation of energy homeostasis

The two major contributing factors to the development of obesity are a change of eating habits leading to chronic nutrient excess with a combination of low exercise levels as a result of our sedentary lifestyle. Other contributing factors are behavioural, environmental and genetic variations, though single gene defects account for no more than 5 % of morbid obesity (Yeo, 1998). With regards to polygenetic influences, evolution may have selected gene variants resulting in a genetic makeup that is designed to maximize survival in times of limited nutrient availability ('thrifty genotype hypothesis') (Neel, 1998). The price for these adaptive genes is continuous weight gain in an environment of excess energy-rich food and lack of exercise (Schwartz, 2004). The self reported calorific intake from 1980 to 2000 may have changed little (NICE, 2001), but today's diet is energy denser and unhealthy food is more readily available, especially in the form of a high fat diet.

Obesity is associated with abnormal adipose tissue biology resulting in ectopic fat deposition and fat inflammation which are explained in detail in Section 1.4.6.. The increase of fat mass also alters secretion of AT products like adipokines which are proteins predominantly produced in AT. Additionally, the brain coordinates energy status through input on short term energy availability and fuel stores from fat reserves. Central response can lead to a change in appetite or influence peripheral metabolism and efferent signals, via the brainstem, can regulate fat mass per se. A yet poorly understood area is the pathophysiology of central pathways of energy homeostasis, some of which may be responsible for the origin of common obesity and possibly T2DM. There are a few rare genetic central feeding disorders involving hypothalamic

popypeptides like proopiomelanocortin (POMC) deficiency (Krude, 1998) and central melanocortin receptor 4 (MC4R) deficiency (Yeo, 1998). Apart from which there is evidence of central resistance to satiety hormones like insulin and leptin (Schwartz, 2005), disturbed hypothalamic sensing of nutrients like glucose and fatty acids (Levin, 1999; Obici, 2002a; Lam, 2005) and other disturbances of the hypothalamic-pituitary–adrenal axis (Koshiyama, 2006). The exact mechanisms of central energy regulation are discussed in more detail in Section 1.3. With the communication between the two organs, the fat and the brain being essential for energy homeostasis and feedback, a disturbance of this crosstalk will ultimately lead to energy imbalance. A defect could involve various sites of the fat brain communication network and studies of the crosstalk may improve the understanding of the pathogenesis of obesity.

1.2. Principles of energy homeostasis

Homeostatic processes seem to have evolved to maintain stable fuel stores in the form of adipose tissue in the face of continuing fluctuations in availability of food. A simple formula of energy input = energy output describes the principle of energy balance by which a stable body weight is maintained. Whereas energy input is derived purely from food, energy output is a sum of following components: basic metabolic rate (BMR), the thermogenic effect of food, and physical activity (Berne, 1998) (Figure 1.2.). The BMR of an adult on an average day is 20-25kcal (84-105 kjoules)/kg of body weight (1kcal= 4184 joules) and is dependent on gender, age, fat mass as well as fat-free mass. BMR can be measured by indirect calorimetry and the use of the respiratory quotient. In a positive energy balance, the energy or calorie intake is greater than the expenditure and the energy excess is stored as glycogen in the liver or triglycerides in fat.

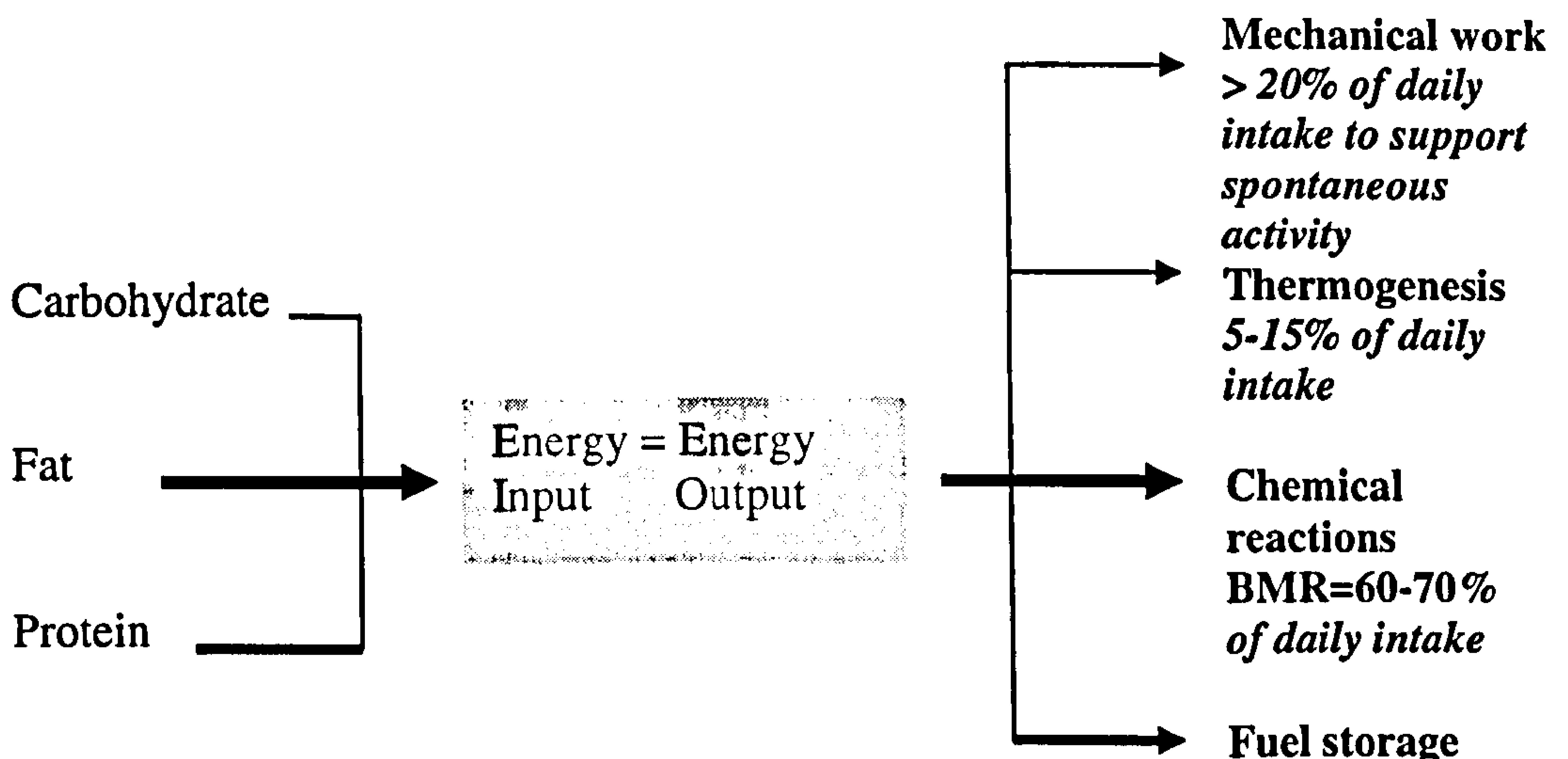


Figure 1.2.: Energy Flow in the human organism. (Modified from Berne, 1998)

In the daily variation of food intake, which includes the overnight fast, there are two distinct phases: the anabolic phase after food intake and catabolic phase from 4 to 6 hours after food intake till the next. The aim of the catabolic

metabolism is to avoid hypoglycaemia with the insulin antagonist glucagon as its key player. The catabolic phase is characterised by mobilisation of fuel in form of substrate from liver, muscle and adipose tissue. This is in contrast to the anabolic phase whereby excess exogenous calories are stored as glycogen in liver or as triglycerides (TG) in adipose tissue. Excess accumulation of TG stores as fat lead to obesity. Whilst adaptive responses in feeding behaviour and other metabolic processes should allow stable body weight, the individual's set point of energy balance appears to be lost in obesity. The reasons for this malfunction are probably multifactorial and there is also the argument of favourable tendency towards energy preservation which challenges the hypothesis of energy homeostasis (Wilding, 1997). However, after a certain surplus of fat tissue has accumulated, the body responds with obesity derived complications and diseases in favour of the existence of a disturbance in energy balance. Identification of a simple defect in one of the energy regulation pathways could potentially lead to much sought after obesity drug development.

1.2.1. Regulation of food intake

Appetite is regulated centrally and modulated through various peripheral hormonal and neuronal signals, as well as nutrients like glucose and free fatty acids (FFA) themselves. The brain integrates visual, olfactory and gustatory sensory stimuli and input from regulators of award and cognitive brain functions with peripheral signals which converge on hypothalamic appetite regulating pathways. Figure 1.2.1. gives an overview of appetite control. Whilst short term signals determine the size of a meal and its frequency, the long term signals determine the fat storage (Griffin, 2000). Of great importance is also the

peripheral appetite regulation by the GI- tract, which includes the pancreas through insulin secretion and adipose tissue (AT) derived products like leptin.

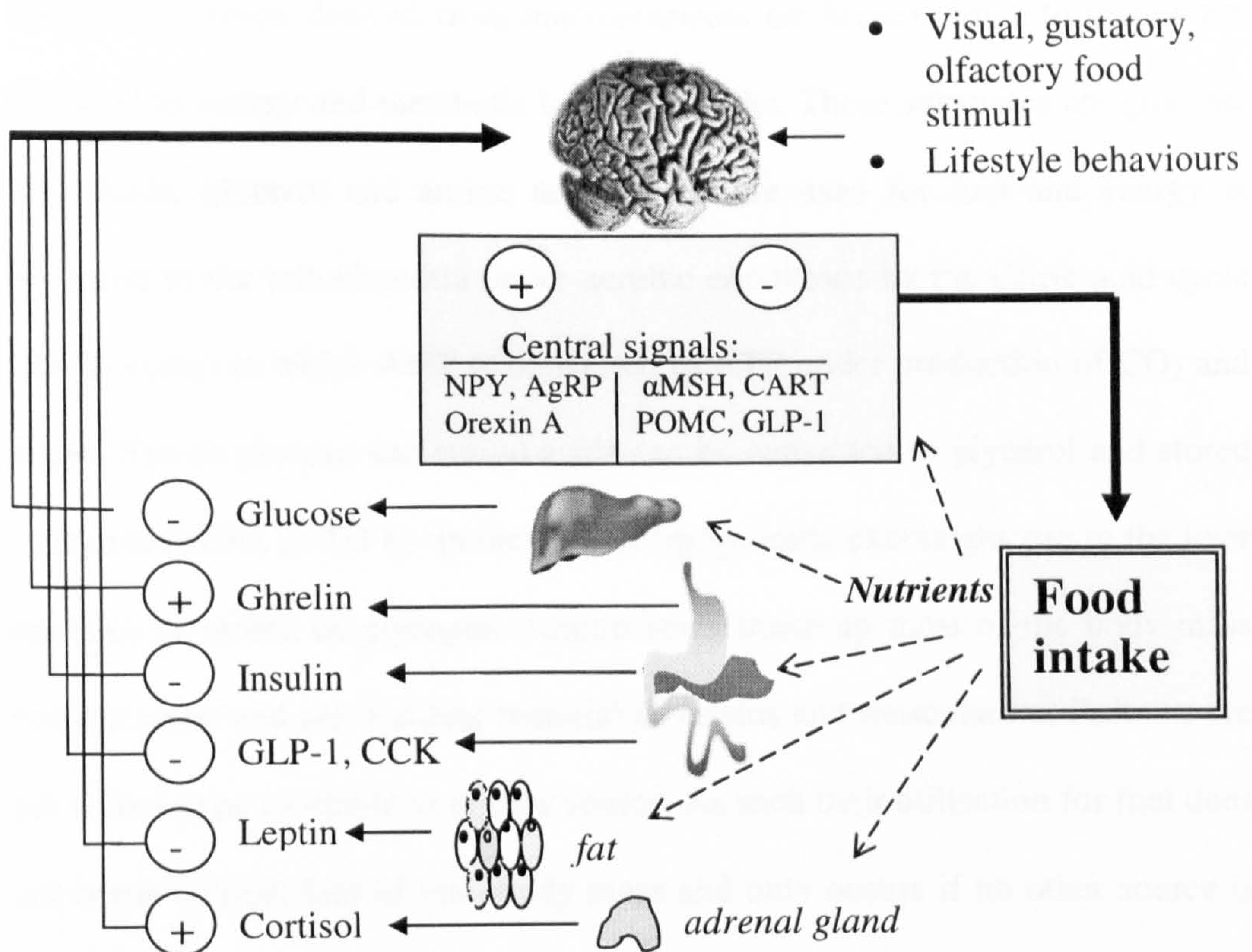


Figure 1.2.1.: Simplified model of appetite control and its feedback mechanisms.

Leptin and insulin share common pathways (Niswender, 2003) and are anorexigenic (appetite reducing), *i.e.*, they are satiety signals. They may be considered as the first identified peripheral proteins which enable crosstalk with the brain. Leptin and insulin mediate their action through central hypothalamic pathways and compete with orexigenic (appetite or hunger stimulating) neuropeptides like ghrelin and neuropeptide Y (NPY) signalling. Their central pathways are dependent on phosphatidylinositol 3-kinase (PI3K), tyrosine phosphatase-1B (PTP-1B), suppressor of cytokine signaling-3 (SOCS-3), adenosine monophosphate (AMP) activated protein kinase (AMPK) and the Janus-family tyrosine kinase- (Jak-Stat) pathway in the periphery and the brain (Sahu, 2004; Schwartz, 2004; Fruhbeck, 2006).

1.2.2. Fuel utilisation and storage

The metabolic pathways are too complex to review in detail, but in essence, complex polymers derived from macronutrients are broken down to monomers and used as energy and metabolic building blocks. These substrates are glucose, fatty acids, glycerol and amino acids. They are used for fuel and energy is produced in the mitochondria under aerobic conditions by the Citric acid cycle (Krebs cycle) in which AMP is converted to ATP under production of CO_2 and water. Excess glucose and amino acids can be converted to glycerol and stored with excess FFA as TG by an increase of lipogenesis; excess glucose in the liver can also be stored as glycogen. Amino acids make up most of the body mass besides water and are building material of organs and musculature. Proteins are not stored to be available as energy source. As such their utilisation for fuel does not occur without loss of vital body mass and only occurs if no other source is available (Frayn, 2006). Fuel storage is enhanced by the anabolic hormone insulin, which inhibits catabolic pathways and is a switch from fat utilisation in the absence of insulin to carbohydrate utilisation in peripheral tissues. Both insulin and macronutrients regulate transcription factors and control gene expression of proteins involved in 'switching' between fat and carbohydrate utilisation. One of such pathways in the liver is via a subfamily of forkhead transcription factors (FOXO), which turn off glycogenolyis and influence lipolysis (Imae, 2003).

1.2.3. Carbohydrate metabolism

Glucose is an obligatory fuel source of some organs; another important source are ketones which can be used as substrate by brain in prolonged starvation. In

normal individuals, fasting glucose levels are kept in a tight range through constant regulation of glucose absorption in the intestine, production by the liver and uptake by peripheral tissues such as muscle and adipose tissue (Saltiel, 2001). Carbohydrates need to be converted to glycogen for storage in the liver and muscle, because of the high osmotic potency of glucose (Frayn, 2006). Depending on the type of tissue and the cellular status of the tissue, its fate within the metabolism varies. Glucose can potentially be oxidized completely to CO_2 and H_2O or incorporated into glycogen or TG for energy storage. Besides its potential use for *de novo* fatty acid synthesis, it can also be converted into non-essential amino acids or partially oxidized to lactate, which may return to the liver for gluconeogenesis, constituting the Cori-cycle (Herman, 2006).

1.2.4. Insulin signalling

Insulin is a key hormone of fuel metabolism, which includes glucose uptake to various cells, but also regulation of gluconeogenesis and glycogenolysis in the liver and down regulation of hepatic glucose output (Michael, 2000). As mentioned previously, insulin influences energy homeostasis in the brain as well as fat. It controls appetite in the arcuate nucleus (ARC) of the hypothalamus and influences adipogenesis as will be detailed in the related sections. Insulin secretion from the pancreatic ß-cell is not only stimulated in response to circulating glucose but also to several gut hormones like glucagon-like peptide-1 (GLP-1). These gut hormones are also known as incretins (see below).

Insulin as well as leptin use the IRS-PI3 kinase pathway for signalling not only in skeletal muscle, liver and adipocytes, but also by the hypothalamus in the regulation of central energy homeostasis (Niswender, 2003). Insulin binds to the

insulin receptor (IR) which is part of the tyrosine kinase receptor subfamily like IGF and insulin receptor-related receptor (IRR). IR is a cell-bound receptor with 4 subunits consisting of two α and two ß chains. Phosphorylation of the IR through insulin binding allows subsequent interaction and phosphorylation of tyrosine residues on molecules of the insulin receptor substrate (IRS) family (Sesti, 2001), which provide docking sites for the Src homology 2 (SH2) domain of phosphatidylinositol 3-kinase (PI3K). PI3K converts the membrane lipid phosphatidylinositol (4,5) biphosphate (PIP2), to phosphatidylinositol (3,4,5) triphosphate (PIP3). This process activates 3' phosphoinositide kinase-1 (PDK-1), which in turn activates protein kinase B (PKB) otherwise known as Akt.

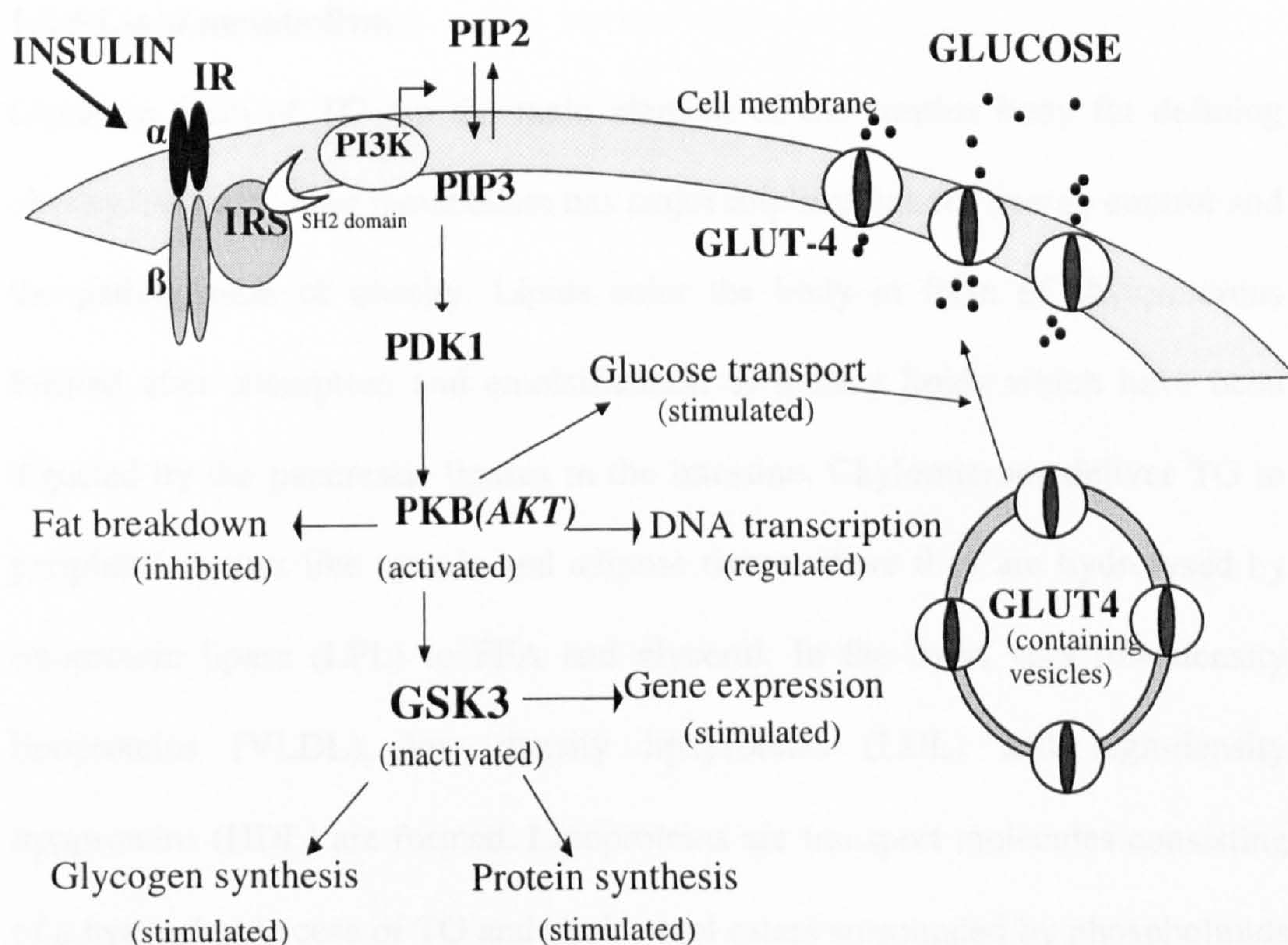


Figure 1.2.4.: Main insulin signalling pathways that regulate glucose metabolism in muscle cells and adipocytes as discussed in the text. Activation of phosphoinositide-3 kinase is a major pathway in the mediation of insulin stimulated glucose transport and metabolism. GLUT-4 is stored in intracellular vesicles. (Based on Cohen, 1999 and Frayn, 2006).

The kinases and enzymes involved in this cascade control a variety of cellular processes which also regulate the insulin mediated glucose transporter 4 (GLUT4) translocation to the plasma membrane (Ono, 2001) and enable active glucose transport into the cells (Figure 1.2.4.). GLUT4 is amongst others expressed in adipose tissue and responsible for insulin-stimulated glucose transport into these tissues (Shepherd, 1999). GLUT4 mediated glucose transport is the rate-controlling step in insulin-mediated glucose disposal which is diminished in insulin-resistant states (Saltiel, 2001) and, paradoxically, down regulated in obesity (Kahn, 1989; Herman, 2006)

1.2.5 Lipid metabolism

Lipids in form of TG are the main element of the surplus body fat defining obesity. As such, lipid metabolism has major implications for energy control and the pathogenesis of obesity. Lipids enter the body in form of chylomicrons formed after absorption and emulsification of dietary lipids which have been digested by the pancreatic lipases in the intestine. Chylomicrons deliver TG to peripheral tissues like muscle and adipose tissue where they are hydrolysed by lipoprotein lipase (LPL) to FFA and glycerol. In the liver, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high-density lipoproteins (HDL) are formed. Lipoproteins are transport molecules consisting of a hydrophobic core of TG and cholesterol esters surrounded by phospholipids and a family of proteins named apolipoproteins (Ganong, 1997). They can be distinguished by the density and indirectly, as lipid is less dense than protein, by their protein content with HDL containing more protein than lipid. Non-esterified fatty acids (NEFA) are the transport form of energy from storage

depots to utilisation sites. NEFA are FA bound to albumin and not esterified to glycerol or any other alcohol group. Its plasma concentrations are determined by the rate of AT lipolysis and cellular uptake. In order to produce energy, FFAs need to be activated in the cytoplasm by acyl-CoA synthetase and enter the mitochondria to be oxidised. The end product of each round of ß-oxidation is acetyl-CoA which enters the Citric-acid cycle, where it is further oxidized to CO_2 to produce energy. The storage of TG in adipocytes will be discussed in the next chapter. Other lipids are phospholipids (constituents of cell membranes) and steroid compounds and are not used for fuel storage. The transport of FFA into cells is less clear than glucose, it is thought to enter partially through diffusion and mechanisms like Acetyl-coenzymeA (CoA) synthase, which esterifies FFA to CoA, enabling ATP dependent intracellular transport, or mediated by CD36 which is distributed in adipose tissue and skeletal muscle (Frayn, 2006). CD36 may be regulated by insulin and exercise and recruited from an intracellular pool similar to GLUT4 for glucose transport.

1.2.6. Fuel sensing

Glucose sensors enable cells to translate the changes in glucose flux and participate in systemic glucose homeostasis (Hermann, 2006). Glucose entering the cell changes gene transcription and modulates signal transduction networks through its metabolites and its sensors. For example, malonyl-CoA is a product of the enzyme acetyl-CoA carboxylase (ACC) and a metabolite implicated in glucose sensing in numerous tissues, including the pancreatic ß-cell and the hypothalamus where it can influence nutrient uptake (Obici, 2003; Hermann 2006). Malonyl-CoA is an allosteric inhibitor of carnitine palmitoyltransferase 1

(CPT1), which regulates the transport of long-chain fatty acyl-CoAs into the mitochondria (McGarry, 1983). In the presence of abundant glucose, malonyl-CoA accumulates and inhibits fatty acid oxidation. Another intracellular nutrient sensor is the enzyme AMPK which is activated through falling ATP levels (adenosine triphosphate) (Hardie, 2003). A decrease of the ATP/AMP ratio leads to AMPK activation and is a signal to switch from anabolic, energy consuming pathways to catabolic, energy producing pathways (Fruhbeck, 2006). The AMPK pathway is found in several tissues and influenced by the adipokines leptin and adiponectin (Havel, 2004). AMPK activation increases FA oxidation through indirect control of malonyl-CoA synthesis and possibly by its degradation, and inhibits TG synthesis (Ruderman, 2003). The activation of AMPK may protect from ectopic fat accumulation (Minokoshi, 2002; Yamauchi, 2002). Hypothalamic AMPK activation increases hyperphagia and induces weight gain (Minokoshi, 2004) which can be inhibited by leptin, insulin and high glucose levels (Fruhbeck, 2006).

1.2.7. Energy homoeostasis and cytokine pathways

Several cytokines and fat derived factors known as adipokines, such as leptin mediate their action through the Jak-Stat pathway (Figure 1.2.7.1), which is able to interact with insulin signalling via the IRS-P13K pathway and indirectly influence energy homeostasis. The enzyme tyrosine phosphatase-1B (PTP-1B), as well as suppressor of cytokine signaling-3 (SOCS3), can terminate signals by cleavage of phosphate from key tyrosine residues of both insulin and leptin (Schwartz, 2004, Fruhbeck, 2006) (Figure 1.2.7.2.).

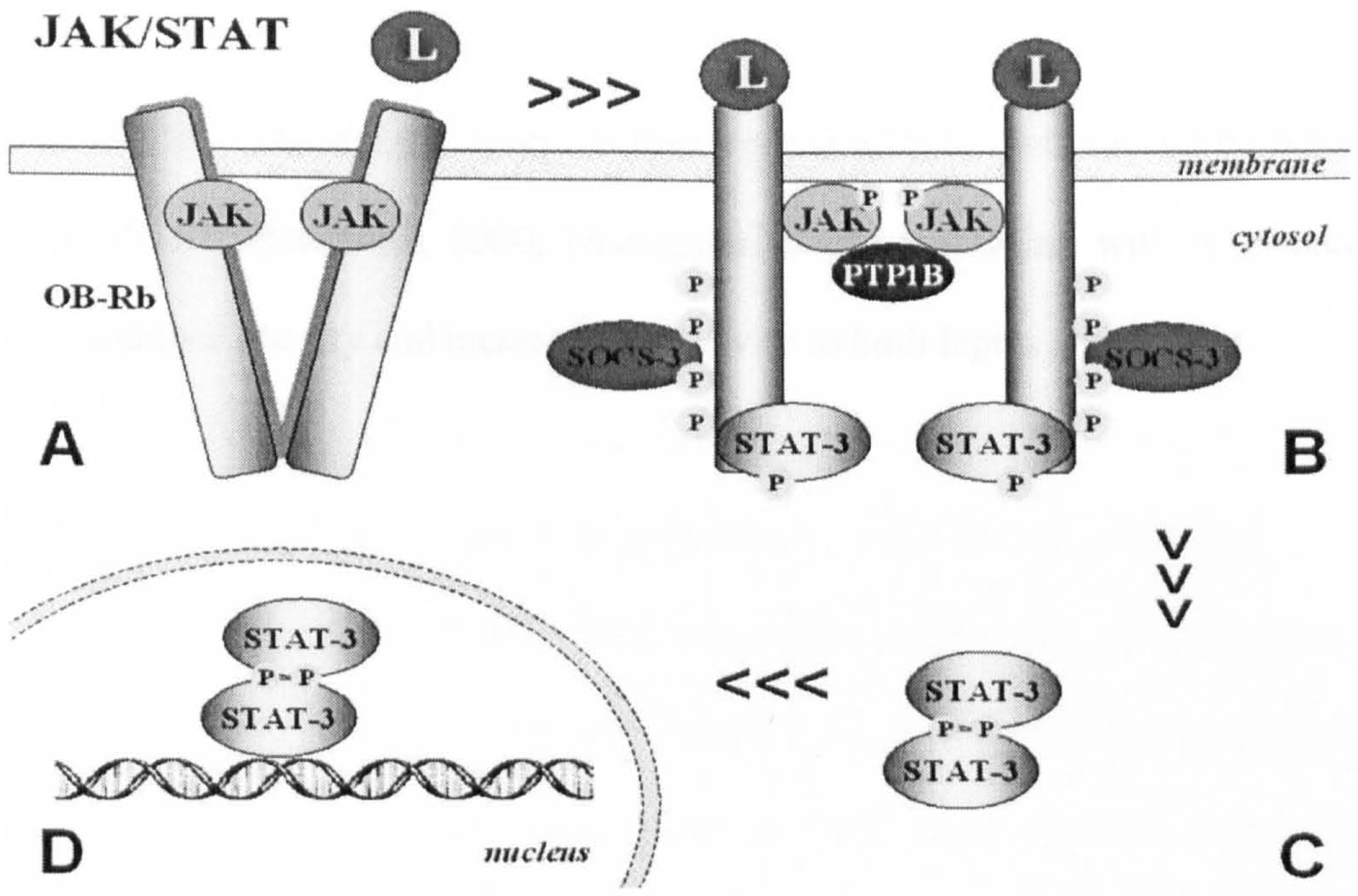


Figure 1.2.7.1.: Jak-Stat pathway. Leptin binding induces receptor dimerization, activating the associated Jaks (Janus-family tyrosine kinases), which phosphorylate the receptor leading to conformational change (**A**). This allows juxtaposition of Jaks, which enables tyrosine-phosphorylation of other Jaks (**B**). Jak activation allows association of Stats in the cytoplasmic region of the receptor. The receptor-bound Stats are phosphorylated by Jaks, dissociate from the receptor and form dimers (**C**). These active dimers translocate into the nucleus where they regulate gene expression (**D**) (Adapted from Fruhbeck, 2006).

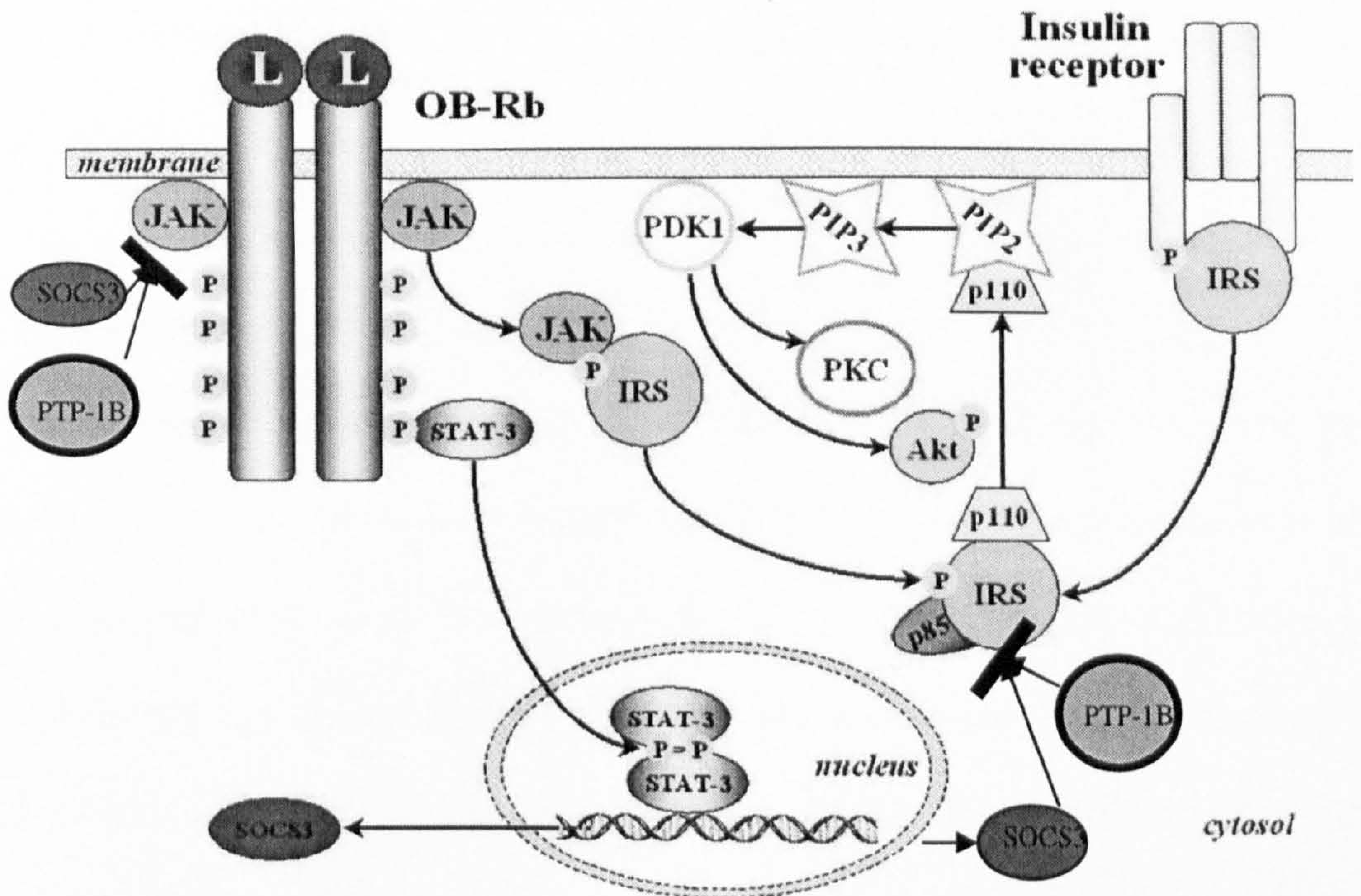


Figure 1.2.7.2.: Jak-Stat and IRS-PI3K interaction. Activation of the Jak-Stat pathway can also activate the IRS-PI3K pathway, presumably due to tyrosine phosphorylation of IRS proteins by Jak 2 (Modified from Fruhbeck, 2006).

The SOCS3 deficient phenotype is thus very similar to that induced by deletion of PTP-1B (Spanswick, 2000; Niswender, 2001) presenting with resistance to diet-induced obesity and increased sensitivity to both leptin and insulin.

1.3. Central regulation of energy homeostasis

The concept of centrally derived human obesity has been first described in 1975, observed by the destruction of the ventromedial region of the hypothalamus (VMH) either by tumours, inflammation, congenital malformation or trauma (Bray, 1975). Similarly, lesions in the paraventricular nucleus of the hypothalamus (PVN) in rats were observed to cause obesity (Tokunaga, 1986) whilst ablation of the lateral hypothalamic area (LHA) was associated with an increased energy expenditure and reduced food intake (Teitelbaum, 1962). Imaging studies with functional MRI or PET have confirmed that taste, olfactory and visual signals as well as nutrients affect appetite and stimulate a diversity of brain centres including cortex, amygdale, area postrema, limbic system and hypothamalus (Tataranni, 2003) of which the hypothalamus, with its many nuclei, appears to have a switchboard function. Information on energy balance from the brain and peripheral organs is collected and further processed in the hypothalamus, which has connections to other higher centres and the periphery.

1.3.1. Hypothalamic anatomy

The brain obtains input on both energy stores and fuel status to control the maintenance of a stable body weight. It is able to respond to afferent signals of energy status by short term regulation, *e.g.* in mediating appetite changes, influencing endogenous glucose production and modulating energy expenditure through mobilisation of fat stores (Schwartz, 2005). This necessitates sensing and translation of peripheral input into anabolic and/or catabolic signals by neuronal circuits which mediate energy regulation. A pivotal role has been

identified for the ARC, which mediates its action through second order neurons to other energy regulating centres of the hypothalamus like the PVN, but also the brainstem via the nucleus tractus solitarius (NTS) which sends efferent vagal signals to the periphery to mediate peripheral energy metabolism. There are two primary populations of neurons in the ARC (Figure 1.3.1.1.); a catabolic or food inhibiting circuit of neurones expressing proopriomelanocortin (POMC)- a melanocortin precursor peptide- and the cocaine-and amphetamine-regulated transcript (CART) (Schwartz, 1997; Morton 2004); and the orexigenic hormone NPY and agouti related protein pathway (AGRP) (Cone, 2001). Whilst the catabolic population is stimulated by leptin, the latter is inhibited; several other hormones and nutrients act through ARC neurons although they may also have direct effects on other brain centres (Schwartz, 2002).

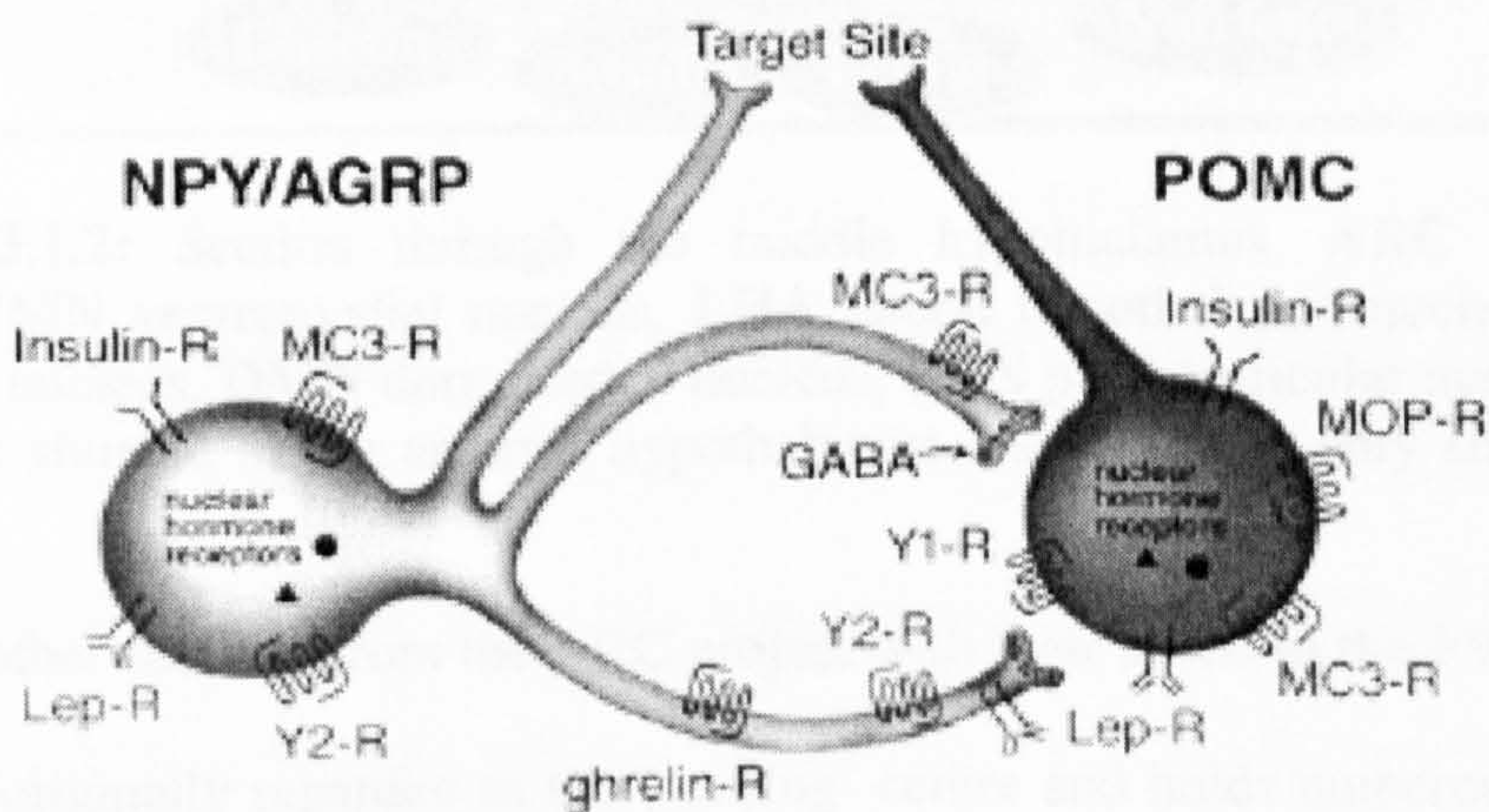


Figure 1.3.1.1.: Hypothalamic appetite regulating neurons in the ARC. The anorexigenic POMC and orexigenic NPY/AGRP neurons are interconnected. Electrophysiological studies show that insulin, leptin, ghrelin, melanocortin and nutrients act on both sets of neurons (Adapted from Cone, 2001).

The anatomical location of the ARC is at the base of the third ventricle above the median eminence, which is especially permeable for certain proteins like insulin and leptin as one of the circumventricular organs of the blood brain

barrier (BBB) (Johnson, 1993). The neuronal connections within the hypothalamus and its communication with other brain areas are complex and not yet fully understood. A simplified model of hypothalamic appetite regulating sites is shown with Figure 1.3.1.2..

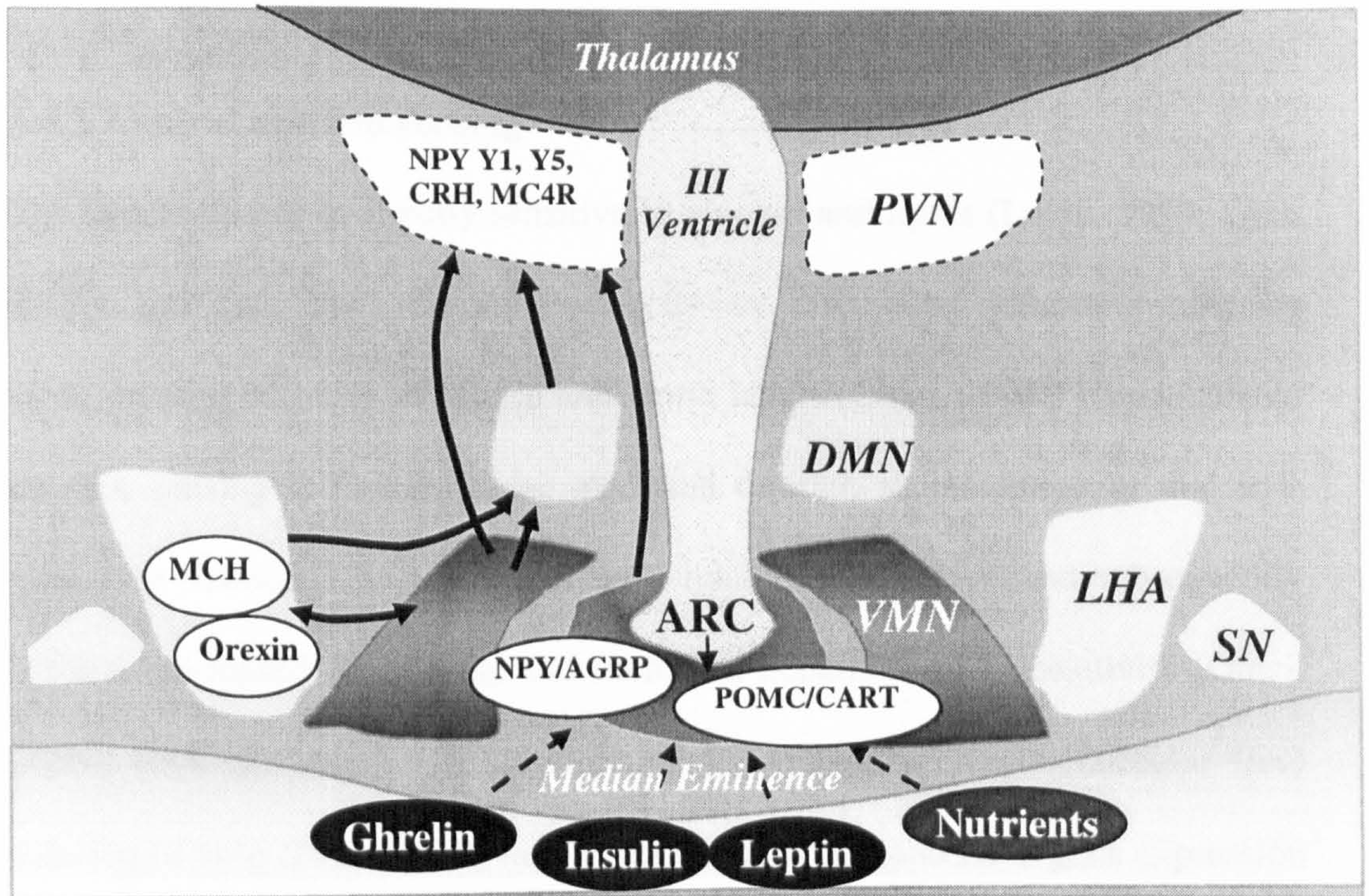


Figure 1.3.1.2: Section through the middle hypothalamus. **ARC** arcuate nucleus, **VMN** ventromedial nucleus, **LHA** lateral hypothalamic nucleus, **SN** supraoptic nucleus, **DMN** dorsomedial nucleus, **PVN** paraventricular nucleus in dotted line situated in the anterior hypothalamus and therefore only shown in relation.

NPY and other neurons from the ARC project with their axons to the PVN. The LHA was originally regarded as the 'feeding' centre and holds numerous NPY neurons and receptors. It contains orexin and MCH expressing neurons and glucose sensitive neurons. It entertains close connections to other areas of the medial hypothalamus and the brainstem. This is thought to allow processing of visceral functions like taste, gastric distension and the sleep-wake cycle (Bernardis, 1996). The VMN is thought to be a key target for the appetite inhibiting hormone leptin (Satoh, 1997), receives input form the ARC and

projects to the NTS and other hypothalamic nuclei like the dorsomedial nucleus (DMN) (Stanley, 2005). The DMN is thought to have an integrative information processing role and collects input from various other nuclei through extensive neuronal connections (Elmquist, 1997).

1.3.2. Central nutrient sensing

The hypothalamus is directly sensitive to glucose and lipids (Levin, 1999; Lam, 2005) and can also respond indirectly to circulating macronutrients by corresponding changes of insulin and leptin levels (Obici, 2003). Hypothalamic nutrient sensing is thought to be mediated through accumulation of malonyl-CoA (see Section 1.2.6.) which in turn inhibits ß-oxidation and subsequently leads to decreased food intake and increased hepatic insulin sensitivity (Obici, 2003). FFA can enter BBB bi-directionally and intracerebroventricular (*icv*) infusion of long chain FFA in rats reduces food intake and NPY gene expression and hepatic insulin sensitivity (Obici, 2002a). Hypothalamic lipid sensing is therefore believed to regulate hepatic glucose metabolism via activation of vagal fibres which supply the liver (Schwartz, 2005). Whilst AMPK activation in the ARC and PVN increases appetite (see above) reduction in AMPK expression reduces food intake and body weight, *e.g.* by leptin, MCR agonists, glucose and insulin whilst the orexigenic AGRP increases AMPK activity (Fruhbeck, 2006).

1.3.3. Proteins with central action on energy balance

A humoral signal that regulates central energy homeostasis should meet the following criteria: its serum levels should be linked with obesity; it should enter the BBB and possess central receptors located in areas of energy regulation

(McMinn, 2000). Two proteins fulfilling these criteria are the important peripheral regulators of carbohydrate and fat metabolism: insulin and leptin. They are key regulators of catabolic cerebral energy homeostasis and convey signals that reduce food intake and increase energy expenditure (Schwartz, 2004).

1.3.4. Central role of insulin

Insulin was the first hormone shown to play a part in central appetite control. Long before the identification of insulin in 1923, Bernard (1854) induced diabetes by puncture of the fourth cerebral ventricle ("piqûre diabétique"). Studies documented that *icv* administration of insulin increased hepatic insulin sensitivity and glucose production whilst reducing food intake (Woods, 1979). This supported Bernard's assumption of a possible central origin of diabetes. Contrary to this and once the cerebral glucose uptake was shown to be independent of insulin (Seaquist, 2001), the brain was considered by most as generally insulin insensitive. However, the determination that insulin entered through the BBB, the presence of insulin receptors and their location in brain areas which control food intake and energy homeostasis outweighed other data (Schwartz 1992a; Baura, 1993). The insulin detected in the adult brain is primarily of pancreatic origin entering across the BBB. Insulin sensing and insulin receptors are located in the hypothalamus (Schwartz, 1992a). Central action of insulin is mediated through the IRS-P13 pathway, (Niswender, 2003), which is the same signalling pathway, which is thought to be defective in the pathogenesis of peripheral insulin resistance (White, 2003); whilst blockade of the hypothalamic insulin receptor results in hepatic insulin resistance and

increased hepatic gluconeogenesis (Obici, 2002c). As such hypothalamic insulin resistance was proposed as a contributor to hyperglycaemia in T2DM (Obici, 2002b), and further supported the central action of insulin.

1.4. Adipose tissue

1.4.1. Adipose tissue morphology

Fat can represent up to 30-40% of body mass. AT is composed of adipocytes and its precursors, a connective tissue matrix, nerve tissue, stromovascular cells and immune cells. Pre-adipocytes are precursors of adipocytes, which are undifferentiated cells and serve as a store for adipocyte recruitment when cell expansion is necessary. The different cell types function as an integrated unit. Endothelial cells form the vasculature; the predominant immune cell is the macrophage with some leukocytes, and usually constitutes 5 -10% of cells; this can increase to up to 40% of the cell population within an adipose tissue depot with diet induced weight gain (Weisberg, 2003). WAT is the main form of fat in the human adult. It serves as a store of substrate/energy but also as support tissue and heat regulator. Heat insulation *per se* is a more typical function of the brown fat in animals, which is richer in mitochondria and enables hibernation. In humans the brown fat which is the prevailing form of the foetus is gradually lost during childhood (Symonds, 2004). WAT is formed before birth but its expansion occurs rapidly after birth, with an increase in hypertrophic growth (cell size) and hyperplastic growth (Gregoire, 1998) (see below). WAT adipocytes are unilocular and have a diameter which ranges from 25-200 microns.

1.4.2. Adipose tissue distribution

There are two general patterns of fat distribution: upper body and lower body obesity. The first type is associated with an increase of subcutaneous (Sc) abdominal and intra-abdominal omental fat and also called central obesity. It is

typical of the fat accumulation in men and thus also known as 'android' or 'male type obesity' (Abate, 1996). In contrast, the obesity pattern in women is typically described by fat accumulation in the gluteofemoral area and thighs and also called 'gynoid type obesity' (Arner, 1998). After the menopause, however, when the site of oestrogen production changes from the ovary to the fat (Björntorp, 1995), women also tend to develop central obesity. This distribution of WAT is critical in relation to the manifestation of obesity related complications like cardiovascular disease and T2DM which is increased in central obesity (Vague, 1980, Clausen, 1996). Central adiposity is associated with an increase in visceral fat mass to which the increased health risk was traditionally attributed (Despres, 1995). However, an argument against its sole contribution in disease risk is its proportion to overall fat mass which can only account for 20% in an obese individual. The remaining 80% are composed of Sc fat and are likely to have a considerable impact on an obese individual's metabolic profile (Abate, 1996; Montague, 2000). However, the catecholamine responsiveness of abdominal cells is greater than in gluteo/femoral regions, which triggers lipolytic activity (see below) (Wahrenberg, 1989) and could explain the increased insulin resistance seen in central obesity (Stears, 2001). As such it is still unclear as to which abdominal fat depot is responsible for the enhanced disease risk (Fain, 2004). Besides the gender difference of fat distribution and the overall higher fat mass in women (Krothiewski, 1983), there are also ethnical diversities. For instance, age and BMI matched people of Asian origin have a much higher percentage of body fat than white Europeans (Deurenberg, 1998; He, 2001). This may contribute to the much higher cardiovascular mortality and risk of T2DM in the South Asian

population(Abate, 2001); a similar fat related cardiovascular risk was also reported in the Afro-Caribbean population (Bhopal, 2002).

1.4.3. Adipose tissue expansion

WAT is unique in its ability to increase rapidly in mass which naturally occurs after birth (Gregoire, 1998) or energy excess. The expansion of fat mass usually entails a combination of an increase of adipocyte cell number (hyperplastic growth) and cell differentiation of pre-adipocytes (collectively called adipogenesis) as well as an increase in cell size (hypertrophic growth). Preadipocytes are derived from pluripotent stem cells which can differentiate into mature adipocytes but also chondrocytes, osteoblasts or myocytes. Thus the process of adipocyte acquisition requires replication and clonal expansion of preadipocytes, which are located in the stromal-vascular fraction of adipose tissue to enable adipose tissue growth (Feve, 2005). The ability of adipocytes to proliferate gives no limits to the extent of adiposity and adipocyte hypercellularity is a classical sign of obesity (Faust, 1984).

1.4.3.1. Adipogenesis

The process of adipogenesis can be divided into the basic stages of growth arrest, cell expansion and cell differentiation. From *in vitro* models, it was derived that the initial stage of adipogenesis or preadipocyte differentiation requires re-entrance of growth arrested cells into the cell cycle, which is a cyclic process that controls mitosis in all eukaryotic systems. One cell cycle composes, the doubling of the genome during the S phase followed by the cellular division during mitosis (M phase). In between the M and S phase are the Gap 1 and 2

(G1 and 2) phases which allow the cells to prepare for DNA synthesis or mitotic division. This is regulated by transcription factors like tumour suppressor retinoblastoma protein (Rb) and E2F, which initiate transcription of further proteins depending on their phosphorylation status (MacDougal, 1995). In addition, contact inhibition represses *in vitro* clonal expansion, arrests cell proliferation and commits the cell to differentiation. However, given the correct hormonal stimuli, contact inhibition is not required for *in vitro* differentiation (Gregoire, 1998).

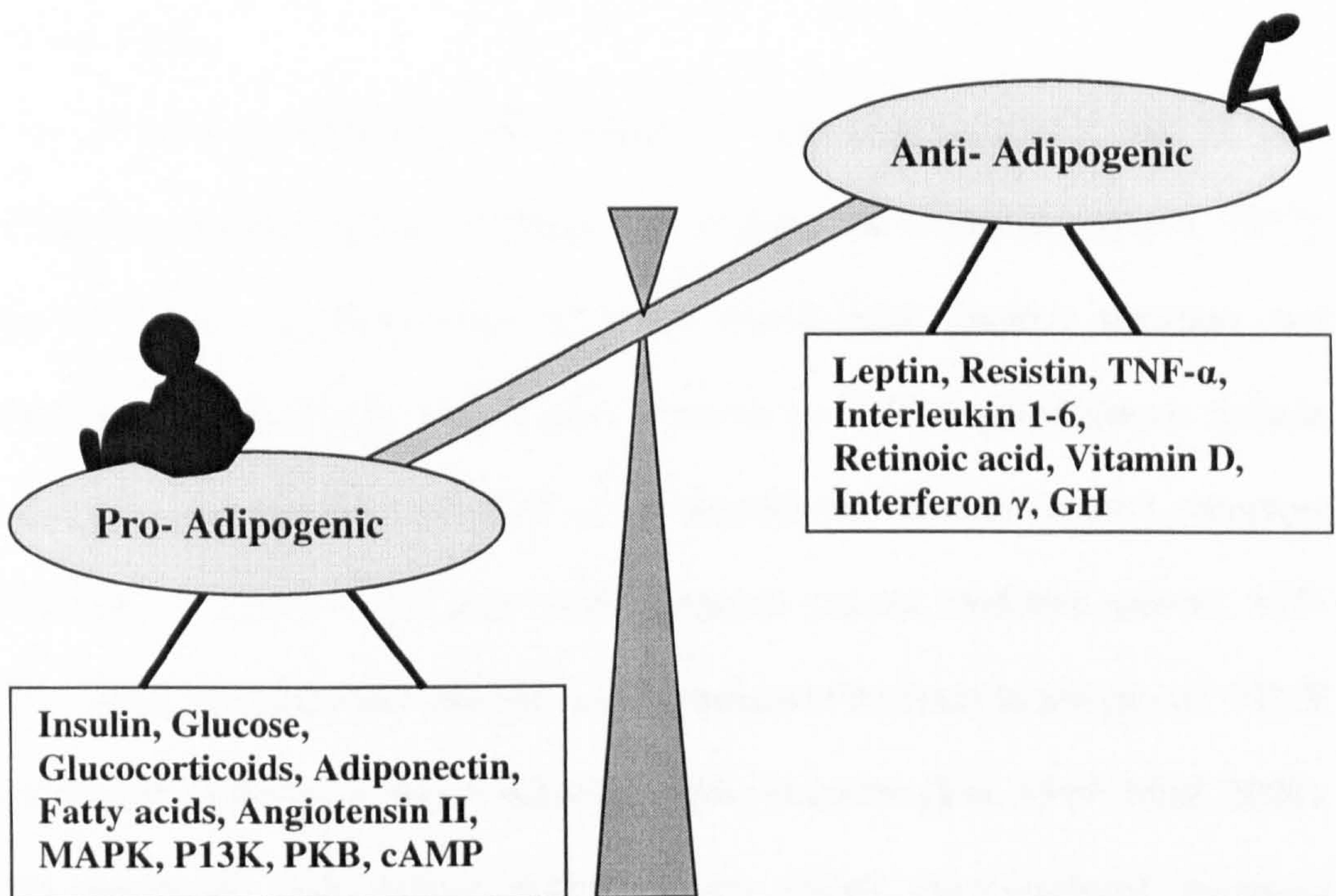


Figure 1.4.3.1.: Balance between pro- and anti-adipogenic environmental signals (Modified from Feve, 2005).

Adipocyte differentiation is coordinated by two main groups of transcription factors CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferators- activated receptor-γ (PPARγ). C/EBPs are induced in the early stages of adipogenesis which initiate further transcription factors and eventually also induce PPARγ, and are thus responsible for maintaining PPARγ expression.

PPARγ belongs to a family of nuclear receptors that initiate the transcription of a multitude of genes and is predominantly expressed in adipocytes (Schwarz, 1997). After their activation by hormonal and nutrient stimuli, both transcription factors, especially C/EBPα, control gene expression of AT specific proteins related to the metabolic actions of insulin such as resistin (Seo, 2003), leptin (Mason, 1998), and GLUT-4 (Gross, 2004) for which they are vital. Furthermore, FFA are endogenous ligands of peroxisome proliferators- activated receptors (PPARs) and regulate their gene expression which can control adipogenesis.

The potential to undergo differentiation is also depot-specific and Sc pre-adipocytes differentiate more easily than visceral pre-adipocytes (Prins, 1997). Figure 1.4.3.1. displays some of many transcription factors, nutrients and hormones that influence adipogenesis. Factors promoting adipogenesis include insulin in part through activation of the insulin like growth factor-1 receptors (IGF-1R), a family of receptors which regulate insulin mediated growth. IGF-1Rs signal primarily through IRS with subsequent increase in activity of PI3-K which is thought to be vital to adipocyte differentiation (Xia, 1999; Miki, 2001). Glucocorticoids can induce differentiation trough glucocorticoid receptors present in preadipocytes which stimulate expression of C/EBPδ (Cao, 1991) and PPARγ (Wu, 1996). In addition, 11β-hydrosteroid dehydrogenase 1 (11β-HSD1), the enzyme responsible for conversion of inactive cortisone to active cortisol sensitises tissue to glucocorticoids and is significantly elevated during adipocyte differentiation with higher expression in visceral adipose tissue (Stears, 2001). Furthermore, there are inhibitors of adipogenesis which hinder the turnover of new adipocytes such as tumour necrosis factor alpha (TNFα),

interleukin 6 (IL6) (Knowler, 1981; O'Rahilly, 1997), and interferon gamma (IFNγ). The mechanism may be indirect inhibition of C/EBPα and PPARγ, *e.g.* by TNFα (Wu, 1999) or mediation through the activators of transcription (STAT), *e.g.* IL6 (Stephens, 1999; Stewart 1999). As some of these cytokines are also secreted by the mature adipocyte (adipokines) their action may be part of an AT growth controlling feedback mechanism.

1.4.3.2. Hypertrophic growth

Fat cell size is primarily controlled by its needs as storage organ of TG and the availability of surplus calories leads to increase in cell size. The storage of post-prandial TG leads to an increase of adipocyte cell size and adipose tissue has an almost unlimited capacity of cellular expansion which leads to obesity (Fruhbeck, 2001). The most abundant molecules in AT are TG, which are broken down to NEFA and transported as a complex with albumin. Plasma NEFA concentrations are derived from AT lipolysis and dependent on the uptake from plasma for oxidation to carbon dioxide or for re-esterification to TG.

1.4.4. Adipose tissue turnover

The most obvious function of AT is the accumulation and storage of surplus substrate as lipids in the anabolic phase in form of FFA, glucose, which can be converted to glycerol for TG synthesis or to FA by *de novo* lipogenesis which is stimulated by insulin. FAs are esterified to form TG for deposit in the adipocyte lipid droplet (Figure 1.4.4.1.). In contrast, the NEFA released from the adipocyte does not convert to carbohydrate, but can be utilised for ketogenesis in the liver. However, the glycerol released from TG breakdown can be substrate for

gluconeogenesis in the liver similar to alanine and lactate; this process is inhibited by insulin and stimulated by glucagon (Frayn, 2006).

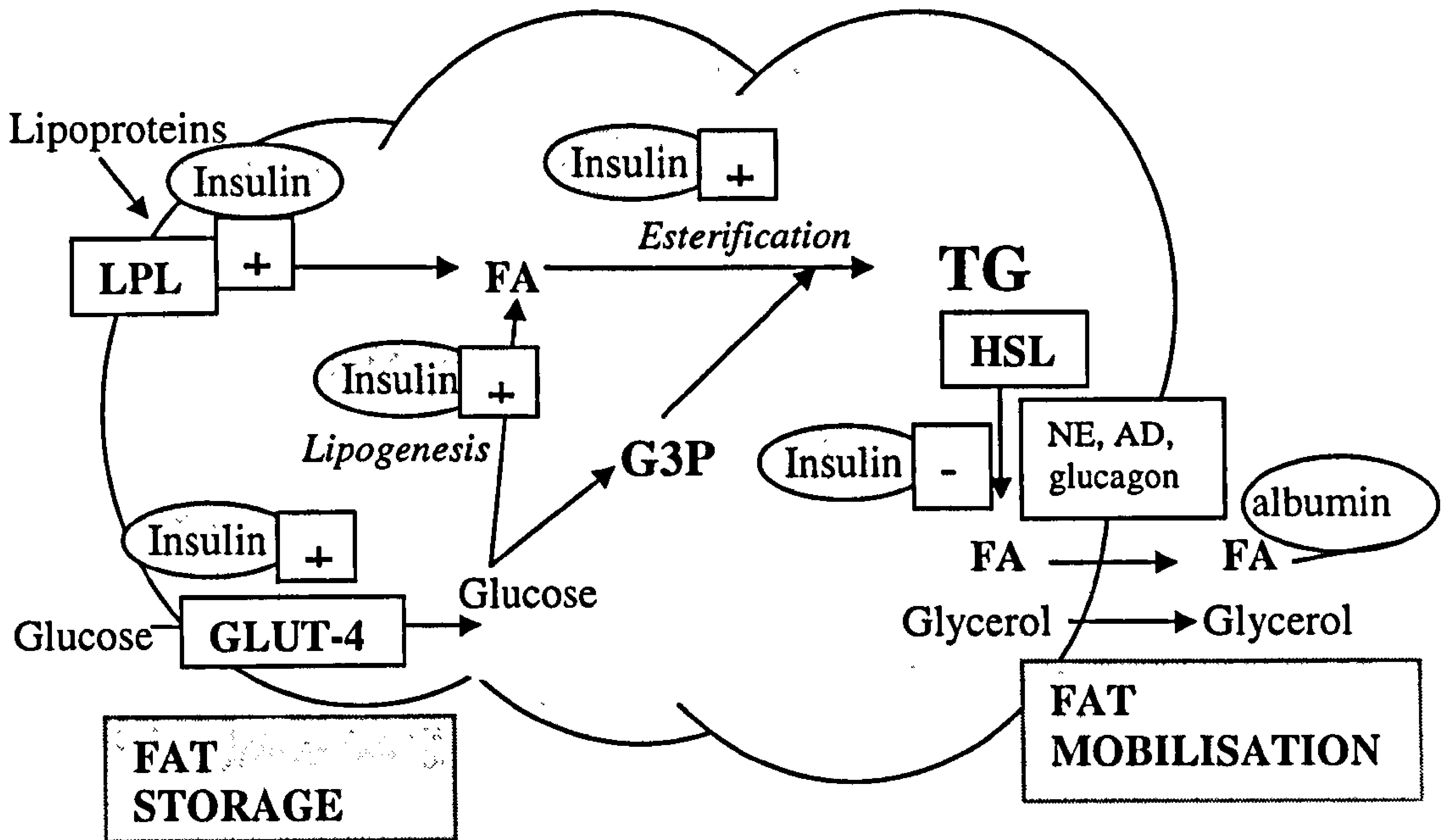


Figure 1.4.4.1: Fatty acid and glucose metabolism in WAT. NE norepinephrine, AD adrenaline (Adapted from Frayn, 2006).

The balance between the sequestering of TG and their eventual release is controlled by two principal processes of lipolysis and lipogenesis. Lipogenesis is defined as the uptake of FFA, mostly from dietary and hepatic sources from the circulation with the help of the enzyme lipoprotein lipase (LPL) to be esterified with glycerol-3-phosphate (derived from glucose by glycolysis) to form TGs. Lipolysis is the sequestering of TG and the release of FFA into the circulation through hydrolysation of triglycerides to glycerol and fatty acids mediated by hormone sensitive lipase (HSL) and relevant isoforms (Ramsay, 1996). LPL is situated within the luminal side of the capillary endothelium and activated by insulin, cortisol (Stears, 2001), oestrogens and its gene expression increased by PPARγ (Frayn, 2006). The resulting fat storage in the underlying form of TG is further enhanced by simultaneous inhibition of HSL by insulin. The

ubiquitously expressed enzyme HSL is activated via phosphorylation by epinephrine, norepinephrine, glucagon, and testosterone. The differential action of sex steroids on adipose tissue turnover may be responsible for the difference in depot fat distribution between male and female subjects (Ramirez, 1997).

Insulin inhibits HSL partially through decreases of cyclic adenosine monophosphate (cAMP) required for HSL activation (Onuma, 2002). Overall insulin is a potent promoter of adipose tissue storage (Figure 1.4.4.2). Increased glucose uptake after induction of GLUT4 allows improved G3P availability for fatty acid re-esterification. The pathways that mediate insulin's metabolic effects diverge downstream and show differential sensitivity to varying levels of insulin. The antilipolytic effect of insulin requires much lower insulin concentrations than stimulation of glucose transport. Hence, even in insulin-resistant states in which glucose transport is impaired, sensitivity to insulin's antilipolytic effect is relatively preserved, resulting in maintenance or expansion of adipose stores (Kahn, 2000).

Other regulators of adipose tissue turnover are nutrients through independent pathways and gene regulation of carbohydrate responsive element binding protein (ChREBP) and sterol responsive element binding protein (SREBP) (Frayn, 2006).

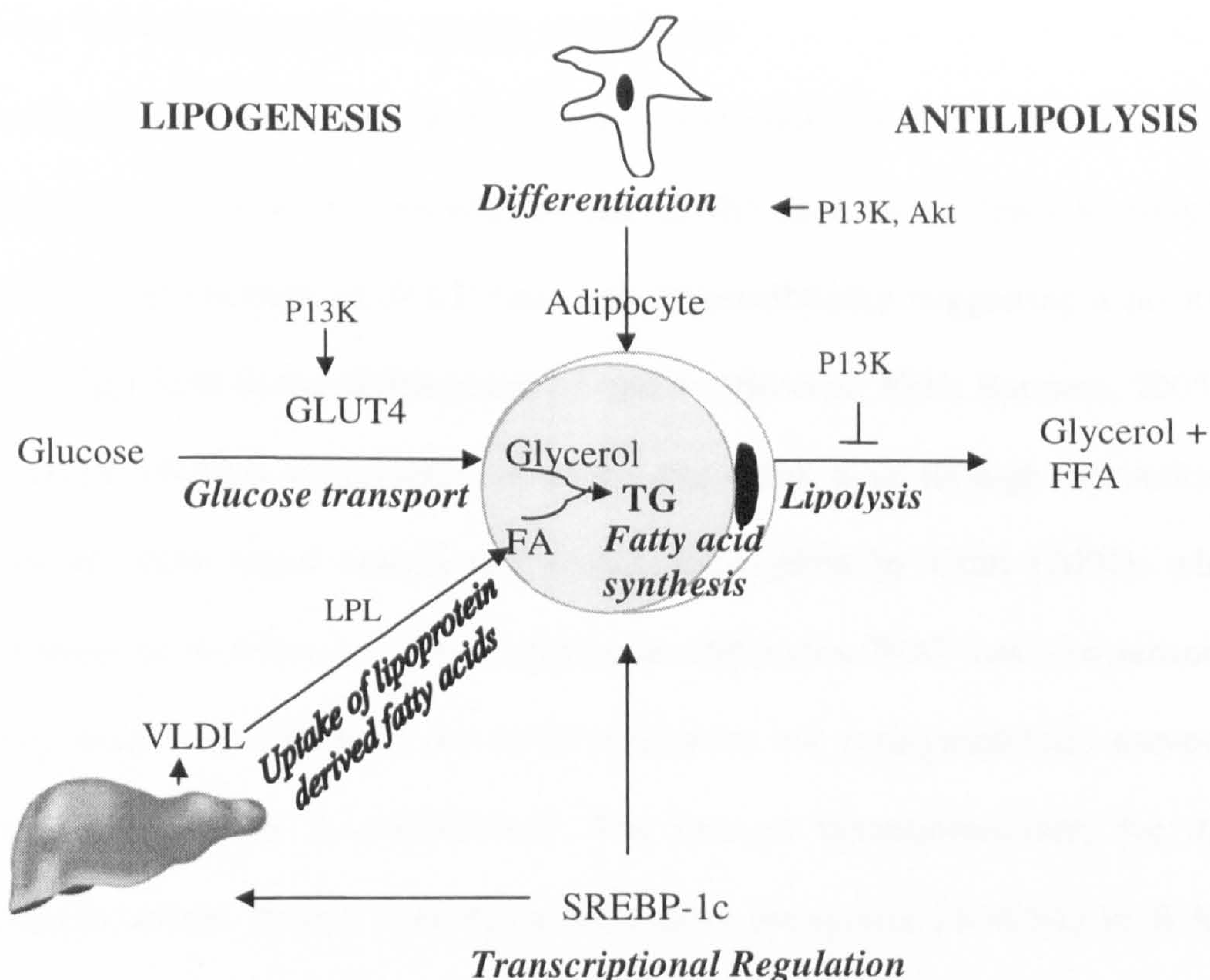


Figure 1.4.4.2.: Insulin as promoter of adipose tissue storage. Many of insulin's metabolic pathways are regulated by PI3K signalling. Insulin promotes adipocyte differentiation, lipogenesis by uptake of glucose and FFA and through induction of transcription factors like SREBP-1c. Insulin also inhibits lipolysis (Adapted from Kahn, 2000).

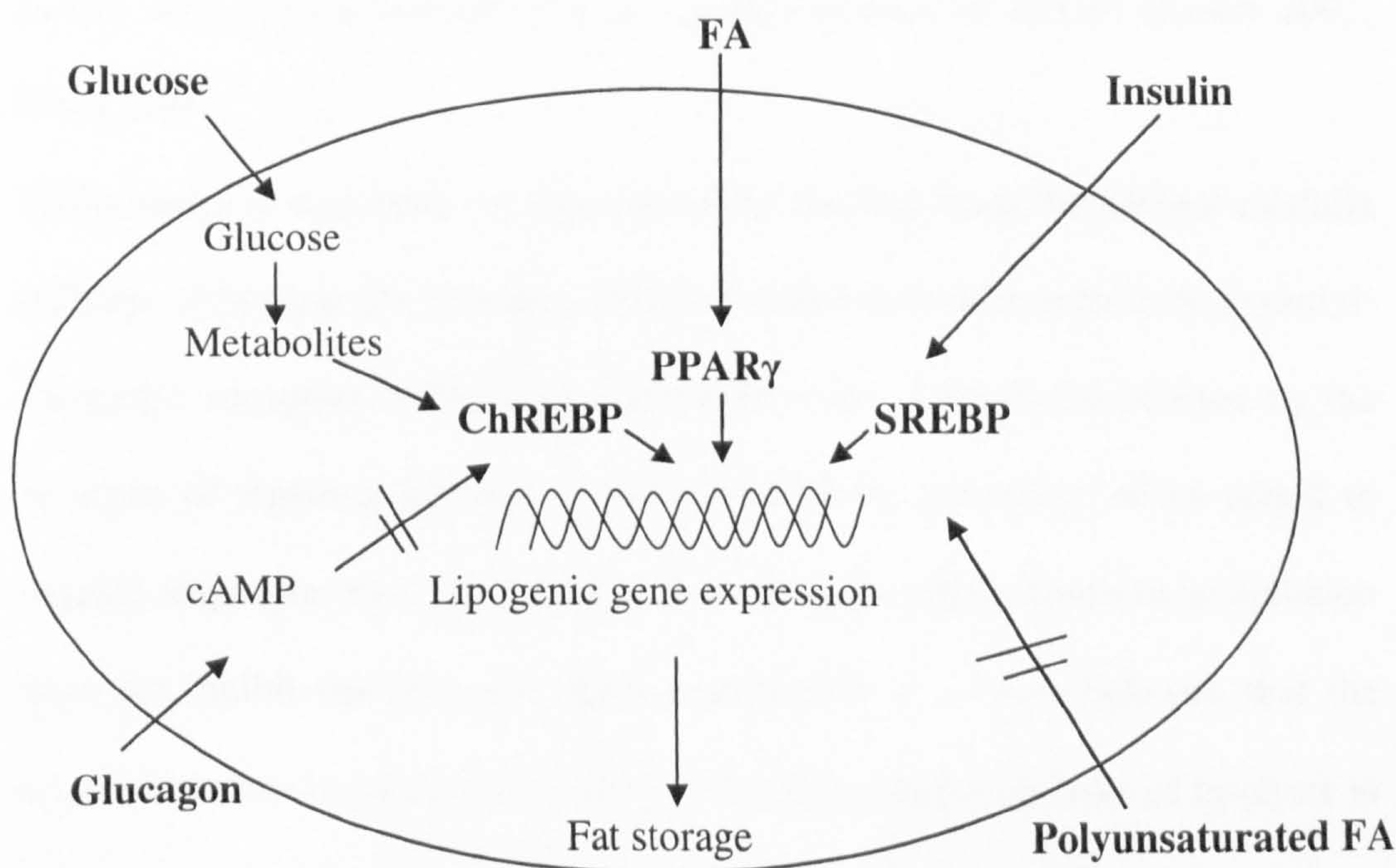


Figure 1.4.4.3.: Nutrients and hormones alter adipocyte gene expression with which they regulate adipose tissue mass (Based on Frayn, 2006).

1.4.5. White adipose tissue and its innervation

Another key regulator of fat homeostasis, especially fat mobilization, is AT innervation. Sympathetic nervous system (SNS) denervation leads to weight gain through increase of WAT mass and hypercellularity suggesting a pivotal role of the SNS in the development of obesity (Bowers, 2004; Bartness, 2005). Evidence of SNS innervation of adipocytes other than through transmitter spillover from blood vessels was finally highlighted by Cinti (2001), who observed nerve fibres in close proximity to adipocytes. WAT has also sensory innervation with CGRP as one of its mediators, but parasympathetic nervous system innervation is controversial. The lack of immunoreactivity for the parasympathetic marker vesicular acetylcholine transporter (VAChT) in WAT argues against this claim (Bartness, 2005). However, studies which have used vagal denervation show changes of fat metabolism with increase of fat growth and induction of insulin resistance. This findings support parasympathetic control of fat and a possible role in the pathogenesis of T2DM (Kreier 2002; Fliers, 2003).

Traditionally it was believed that adrenaline derived from the adrenal medulla (Hillarp, 1954) was the principal stimulant which activates membrane bound ß-adrenergic receptors of WAT to activate lipolysis. This was supported by the evidence of rigorous lipolytic activity induced by adrenaline when added to isolated adipocytes *in vitro*. Based on the observation that adrenal demedullation does not inhibit lipolysis and lipid mobilisation, it is now believed that the neurotransmitter noradrenaline (NA) acts as the principal initiator of lipolysis in WAT mediated through control of cerebral sympathetic innervation and its postganglionic release (Bartness, 2005). There are marked differences in adipose

tissue from humans and rodents, not only by the lack of BAT but also differences of receptor type and distribution in WAT. As such, the predominant catecholamine receptor in human adipose tissue is the ß-2 adrenoceptor as one of four G-protein-coupled adrenergic subtypes (α-adrenoceptor and ß-1,2,3 adrenoceptor) (Arner, 1996; Sennitt, 1998; Rayner, 2001).

The human WAT shows depot specific innervation (Dodt, 1999) with higher lipolytic activity in visceral fat (Hoffstedt, 1997). The fact that Sc adipocytes possess more α_2- and fewer ß-adrenoceptors than visceral fat cells explains in part the lower lipolytic capacity of Sc tissue (Mauriege, 1987). Also, there is a gender dimorphism in lipolytic activity and the typical male fat distribution is in part related to a greater α_2-adrenergic activity and resistance to NA in the upper body combined with an overall reduced lipolytic activity (Lafontan, 2000; Ramis, 2006).

The turnover of NA in WAT increases with cold exposure (Garofalo, 1996) and fasting (Migliorini, 1997), which in turn decreases the adipokine leptin; as such, this implies a tight connection of SNS and leptin regulation. SNS blockade, *e.g.* by use of α –methyl-p-tyrosine (α-MPT)- an inhibitor of a rate limiting step in catecholamine synthesis increases circulating leptin levels and leptin gene expression. Not surprisingly, the observed leptin increment in obesity is associated with reduced sympathetic innervation (Rayner, 2001). Conversely, as part of a feedback regulation, central leptin can stimulate sympathetic activity in WAT (Rayner, 2001) (Figure 1.4.5.)

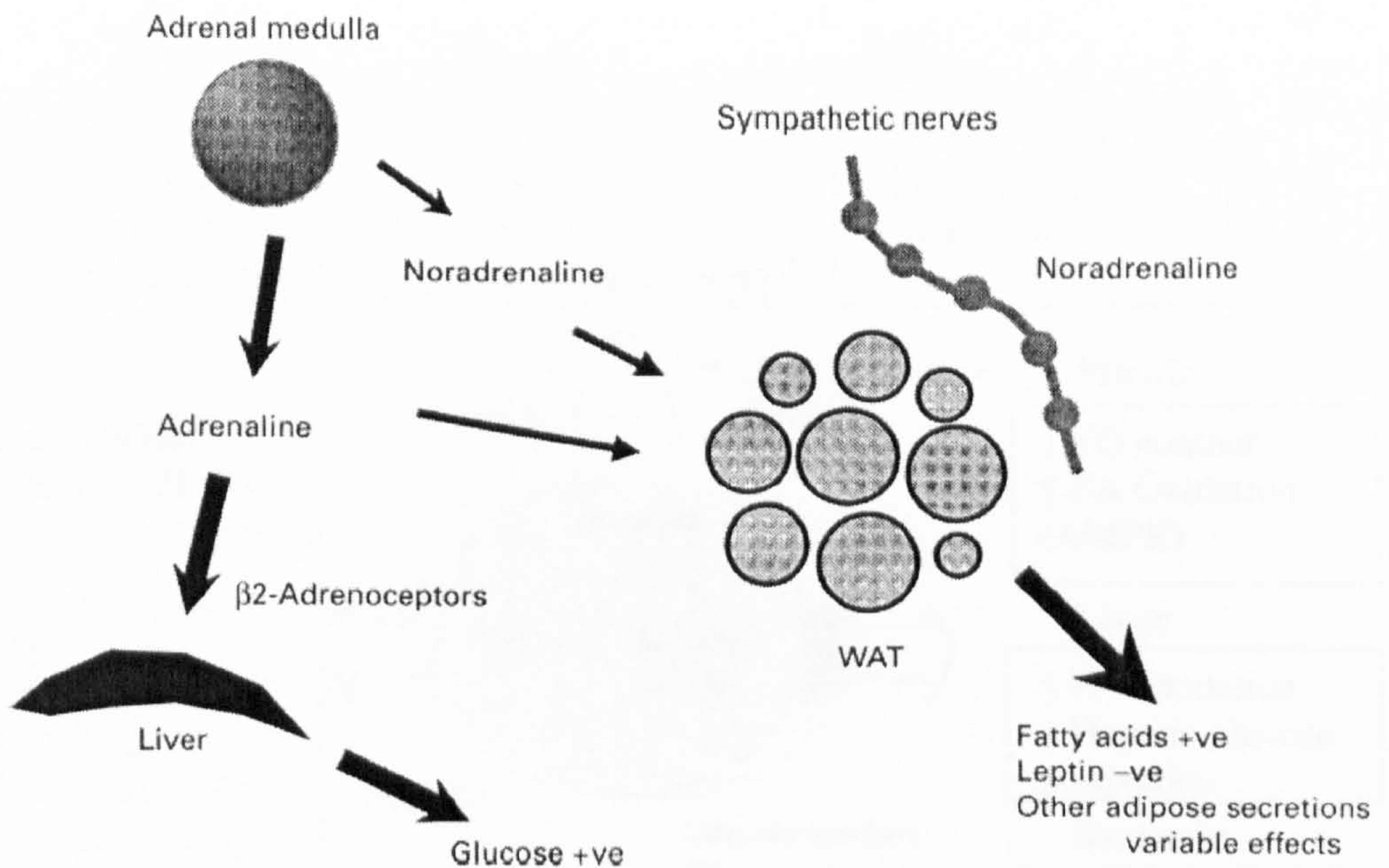


Figure 1.4.5.: Sympathetic regulation of WAT: NA stimulates ß-adrenoceptors, which leads to mobilisation of free fatty acids and inhibits the secretion of leptin. The adrenal medulla derived adrenalin stimulates ß-adrenoceptors in the liver which raises blood sugar, but may only have little influence on WAT other than at times of hypoglycaemia (Adapted from Rayner, 2001).

1.4.6. The endocrine function of adipose tissue

With the discovery of the first adipokines it became apparent that fat is able to exert endocrine effects and influence other systems. The pleiotropic functions of AT thus comprise autocrine (influence on same cell type), paracrine (local involvement of other cell types) and endocrine effects. Through their presence in circulation they can influence the regulation of glucose, blood pressure, regulate systemic inflammation and central appetite and mediate feedback on energy homeostasis. Figure 1.4.6. gives a simplified overview of the diverse systemic actions of adipokines.

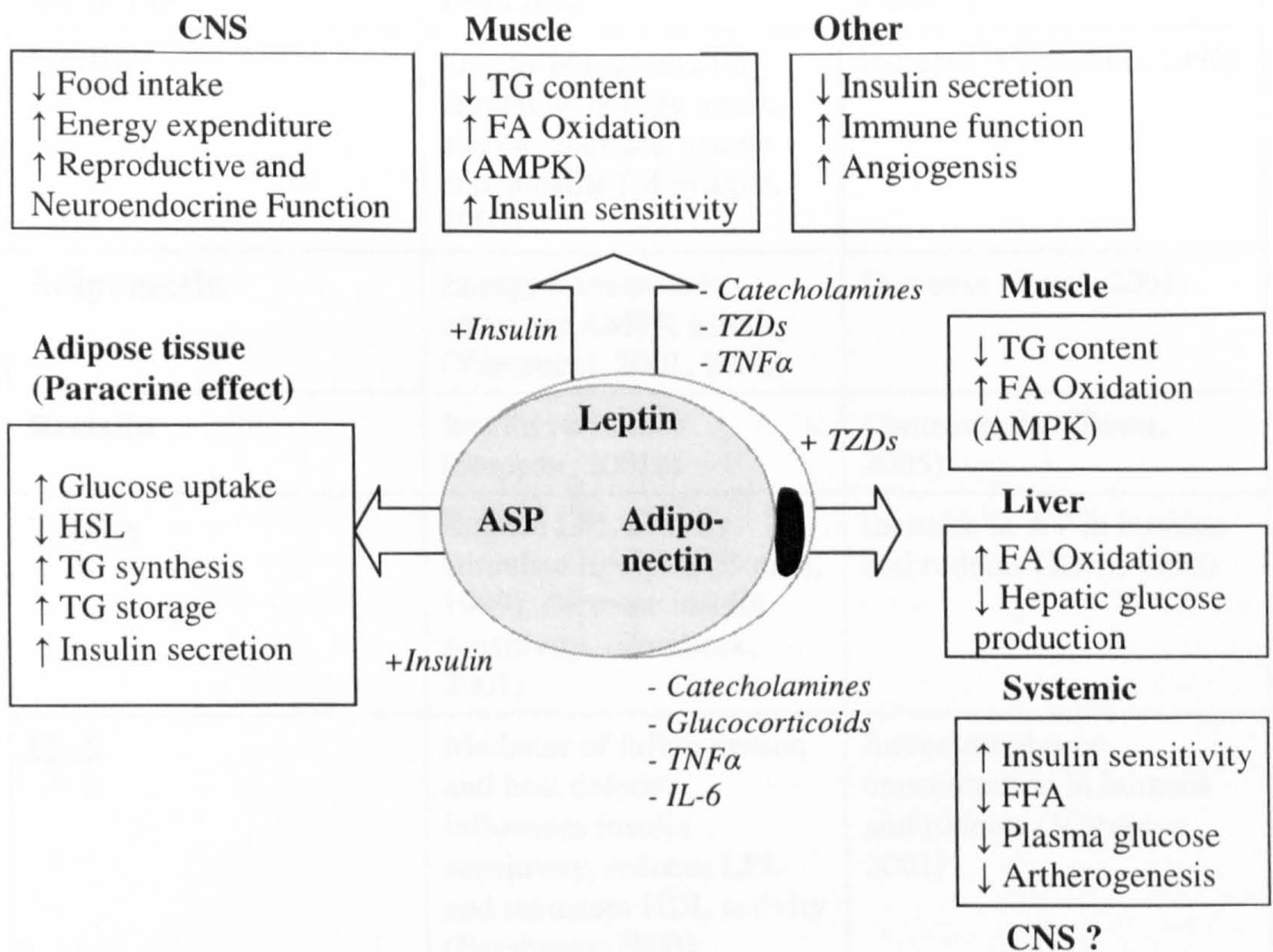


Figure 1.4.6.: Pleiotropic effects of leptin, adiponectin and ASP (Adapted from Havel, 2004).

The adipokines relevant to the topic and these studies are leptin, adiponectin, resistin which are discussed later in separate sections. Additionally, the inflammatory adipokines, *e.g.* interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), have been under much scrutiny and have been associated with the state of subclinical inflammation found in obesity (Pickup, 1997). Most of these inflammatory cytokines are not AT specific but are also produced by macrophages and only a third of circulating human IL-6 is thought to be derived from mature adipocytes within AT (Mohamed-Ali, 1997). TNF-α and IL-6 have been associated with cachexia, reduced fat storage and T2DM (Stears, 2001); they reduce LPL activity (Greenberg, 1992) and stimulate lipolysis (Nonogaki, 1995; Botion, 1999). The increase of lipolytic activity can contribute to elevated systemic NEFA (Makino, 1998).

Adipokine	Function	Obesity
Leptin	Energy homeostasis by regulating energy intake and expenditure, satiety and appetite (Montague, 1997)	Increase (Considine, 1996)
Adiponectin	Energy homeostasis, enhances AMPK activity (Yamauchi, 2001; 2002)	Decrease (Hotta, 2001)
Resistin	Insulin resistance (Steppan, 2001a)	Controversial (Tovar, 2005)
TNF-α	Reduce LPL activity, stimulate lipolysis (Botion, 1999), decrease insulin sensitivity, (Fruhbeck, 2001)	Increase in AT in humans and rodents (Kern, 1995)
IL-6	Mediator of inflammation and host defence, influences insulin sensitivity, reduces LPL and increases HDL activity (Fasshauer, 2003)	Increased plasma concentration in humans and rodents (Vozarova, 2001)
ASP	Increases TG synthesis (Cianflone, 2003)	Increased (Sniderman, 2000)
Angiotensinogen	Regulator of blood pressure (Stears, 2001)	Increased (Stears, 2001)
PAI-1	Potent inhibitor of fibrinolytic pathway (Fruhbeck, 2001)	Increased in the metabolic syndrome and T2DM (Juhan-Vague, 1997)

Table 1.4.6.: Adipokines. Summary of relevant adipokines by review of literature as referenced.

Other adipokines are the adipokine acylation stimulating protein (ASP) which is purely adipocyte derived (Sniderman, 2000) and plasminogen activator inhibitor-1 (PAI-1), which is also produced by other cells (Alessi, 1997). ASP increases the efficiency of adipocyte TG synthesis, inhibits HSL mediated lipolysis (Cianflone, 2003) and enhances the postprandial lipid clearance (Murray, 1999). Whilst ASP levels are higher in obese subjects (Sniderman, 2000) the role of ASP in the pathogenesis of obesity is uncertain. PAI-1 is a key inhibitor of fibrinolysis and found in higher levels in cardiovascular disease

(Juhan-Vague, 1996). Angiotensinogen as well as angiotensin-converting enzyme (ACE) are also widely expressed in human AT, higher in visceral fat, increased in obesity and associated with obesity related hypertension (Stears, 2001). Other AT derived proteins are retinoids (Tsutsumi, 1992), oestrogens and some more recently discovered adipokines visfatin, obestatin and nesfatin. The function of these adipokines are summarised in Table 1.4.6.. Adipokines can take part in central energy regulation, e.g. leptin, or influence insulin secretion and sensitivity (Pischon, 2004; Harris, 1998). As such a dysregulation of adipokine function could contribute to the development of obesity (Rajala, 2003).

1.4.7. Dysregulation of adipose tissue function

There are at least two disease entities with common features attributable to a dysfunctional fat regulation. They are characterized by 'too little and too much' fat mass. The abundance of AT is characterized by the obese phenotype and the opposite by the complete or partial loss of AT is found in lipodystrophy or lipoatrophy. Lipodystrophy usually result from rare genetic disorders with marked pheno- and genotypic heterogeneity, but can also be caused by immune disease or be drug-related, *e.g.* a consequence of retroviral therapy in patients with HIV infection. These patients suffer from metabolic complications similar to those found with obesity, such as insulin resistance/T2DM, hepatic steatosis, hypertriglyceridemia and leptin deficiency, whilst leptin therapy in these patients partially reverses the insulin resistance and hyperlipidaemia (Lee, 2006). Similarly, mice with surgical removal of fat also develop insulin resistance (Weber, 2000).

Another feature of obesity is ectopic fat accumulation which is accumulation of excess TG in non-adipocytes. Non-AT is not adapted for TG storage which leads to steatosis, intramyocellular lipid accumulation and β-cell lipotoxicity (Shimabukuro, 1998) and lipid induced cell death (lipoapoptosis). Failure to confine surplus NEFA to WAT and increased lipolysis can have the consequence of chronically high circulating NEFA levels and is thought to be diabetogenic (Unger, 2003; Makino, 1998). These are toxic to the pancreatic ß-cell (Mason, 1999) and impair insulin signalling in liver and muscle (Dresner, 1999; McGarry, 2002). Whilst the causality of NEFA and insulin resistance is not without controversy and some studies claim the elevation of NEFA is rather a consequence of insulin resistance and a response to high sympathetic tone (Byrne, 1999).

1.5. Crosstalk of adipose tissue and the brain

Adipokines are secreted into the systemic circulation and can mediate endocrine actions through widely expressed receptors. This enables them to communicate with various organs including the CNS (see above). The concept of a hypothalamic-adipocyte axis was described as early as 1991 by Roncari (1991). Most recent theories of the fat-brain axis assume that the same appetite regulating molecules and/or their receptors are expressed in both sites: the hypothalamus and the adipocyte and that they are able to modulate adipogenesis (Shimizu, 2005).

1.5.1. The blood-brain barrier and blood-CSF barrier

To enable a circulating protein to be a mediator of central action it needs to be able to cross the blood-brain or blood-cerebrospinal fluid (CSF) barrier. The tight junctions between the cerebral endothelial cells at the blood–brain interface (BBB) and between choroid plexus epithelial cells (blood–CSF barrier) regulate the entry of molecules (Saunders, 1999). CSF is produced by the choroid plexus, which is a highly vascularised paraventricular 'cauliflower' like structure of convoluted pia mater. The adult human CSF volume is about 120 -150mL with a turnover of about 5 hours. It is produced with a rate of about 21mL/hr and considered to be constant within a normal intraventricular pressure range (Katzmann, 1970). After circulating from ventricles to the spinal canal and over the cerebral hemispheres, it eventually drains into the venous blood absorbed by the arachnoid granulations of the superior sagittal sinus. Production and absorption through the choroid plexus are at a steady state and movement of molecules within the CSF enhanced by microvilli of the outer membrane of the

choroids by the epididymal cells. The epididymal cells have tight junctions and allow only selective entry into the CSF. A high gradient can therefore be maintained between the choroid plexus vessels and CSF, allowing only particular lipophilic molecules to enter (Saunders, 1999); with this it has a dynamic regulatory role of the BBB barrier in the entry of circulating proteins; the presence of a protein in the CSF therefore assumes a certain role and function. A steady state and equalisation of brain/plasma and CSF/plasma ratio is not achieved because of the 'sink effect' of CSF entertained by its continuous turnover, which reduces the steady state concentration of molecules penetrating into CSF and brain with the effect being greater with slower penetrating molecules of larger radius and more lipid-insoluble molecules (Davson, 1969; Saunders, 1999). Proteins can enter the CSF from the blood or the brain. Brain derived proteins enter into the CSF primarily through the ventricular and cisternal space and their concentration between normal ventricular and lumbar spine is decreasing. This is in contrast to the blood derived proteins which are higher in the lumbar region (Reiber, 2001). The kinetics of the actual protein transport across the BBB are poorly understood. Entry is possible by simple diffusion, facilitated diffusion or by receptor mediated transcytosis (Saunders, 1999) as proposed for the insulin blood brain transport by Baura (1996). Insulin transport has recently received much attention in the search of a vector to enable entry of drugs into the CSF (Boado, 2007). Tanycytes, which are bipolar cells bridging the cerebrospinal fluid (CSF) to the portal capillaries may link the CSF to neuroendocrine events (Peruzzo, 2000).

The human ARC in the adult brain is separated from the peripheral circulation. It is in close communication with the median eminence, which is especially

permeable for some proteins and is one of the circumventricular organs which have permeable fenestrated capillaries (Johnson, 1993).

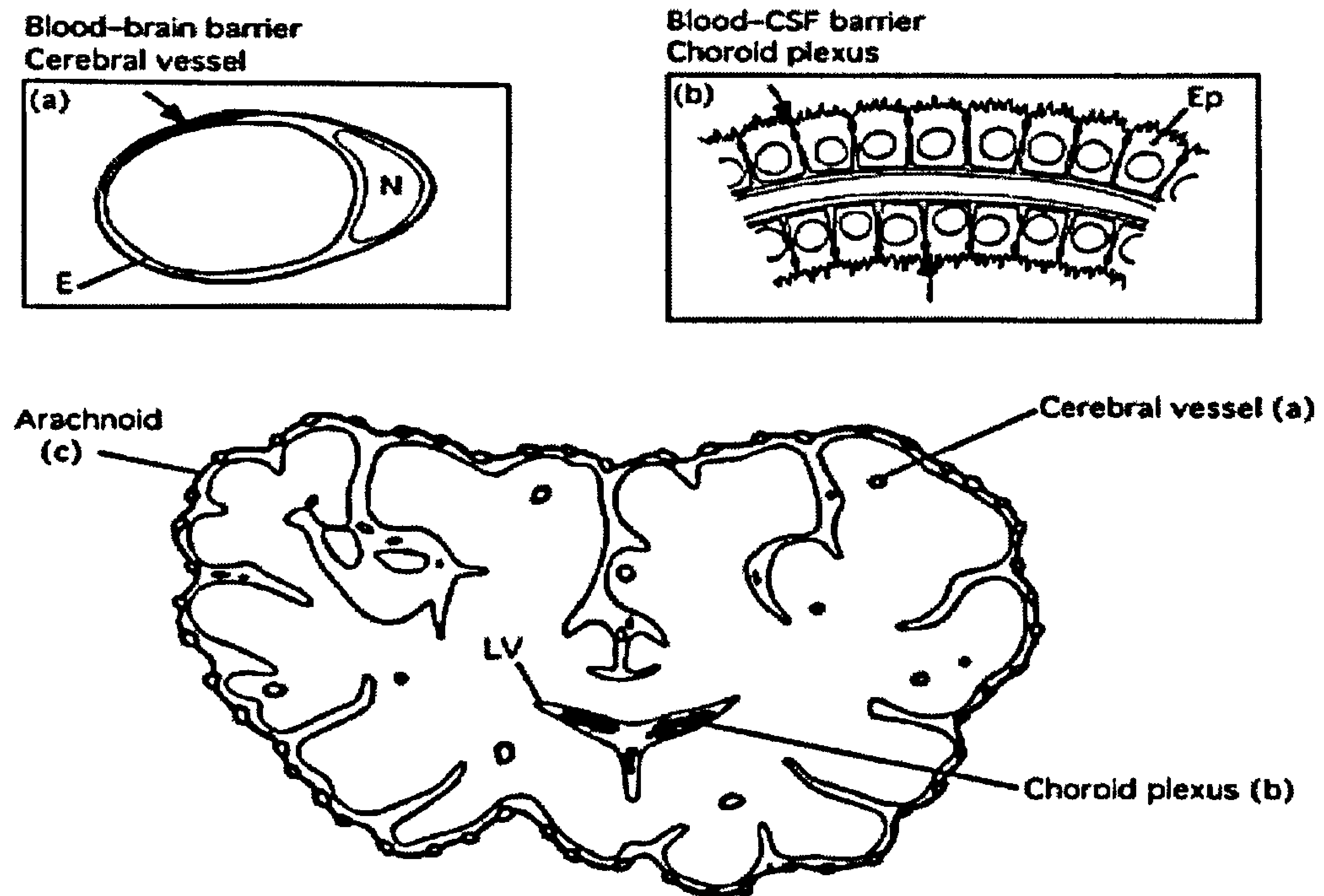


Figure 1.5.1.: The blood brain barrier (a) Cerebral endothelial cell, tight junction (arrow); (b) choroid plexus epithelial cell (Ep) tight junction; (c) arachnoid tight junction (adapted from Saunders, 1999)

The appetite regulating proteins insulin and leptin enter the CSF through saturable transport (Baura, 1993), whilst NPY, polypeptide P (PYY) and GLP-1 enter the BBB through non-saturable mechanisms similar to diffusion (Kastin, 1999; Nonaka, 2003; Kastin, 2002). Experimental methods testing the entry of these proteins typically involve intravenous injection of the radioactive labeled protein in question, vascular depletion studies and estimation of the effect of additional unlabeled protein on the total radioactivity through uptake of the protein in the brain parenchyma (Nonaka, 2003).

The BBB can be affected by several disorders which have been intensively studied in connection with penetration of pharmacological agents. Of particular

interest is the increased permeability of the BBB for insulin in experimentally induced diabetes (Banks, 1997), a possible disturbance of BBB with hypertension, cerebral ischemia and Alzheimers' disease (Banks, 1996). The changing albumin quotient in many neurological diseases however, is an indicator of changing CSF flow rate rather than morphological "leakage" of the BBB (Reiber, 2003).

1.5.2. Transport from CSF to hypothamic sites of appetite regulation

In understanding the mechanisms of transport from the CSF to the hypothalamus brain-perfusion studies in rodents have suggested a differential regulation of the CNS entry of proteins depending on the brain area in question (Zlokovic, 2000). For instance, despite extensive research of leptin transport to the ARC as site of its action, leptin entry is poorly understood. Many neurons of the hypothalamus nuclei are isolated from the BBB circulation yet express a high number of leptin receptors (Baskin, 1999). Hypothalamic neurons are extremely sensitive to binding of leptin, yet due to too low maximal binding capacity the exact binding constants are difficult to determine (Corp, 1998). Hypothalamic leptin uptake is up to 37 times faster than in other brain regions and appears to be mediated by high affinity transporters with K_M of 0.2ng/mL (Zoklovic, 2000). Whilst leptin receptors are found at the BBB (Bjorbaek, 1997), there is no evidence that they are able to assist the transcytosis of this protein (Zlokovic, 2000). It is thus yet unclear whether leptin transport to its hypothalamic sites of action is CSF derived, blood derived (Banks, 1996) or through diffusion from the median eminence of the hypothalamus which lacks the BBB (Zlokovic, 1995; Zlokovic, 2000).

1.5.3. Central action of adipokines: 'The fat-brain axis'

The adipokine leptin signals satiety through interaction with neurons in the ARC leading to reduced food intake. Its central action is well described and will be detailed in Section 1.6.2.. However, little is known about the potential central action of the adipokines adiponectin or resistin in humans and there is little evidence from rodent studies. Table 1.5.3. gives an overview of central effects of adipokines and proteins involved in the regulation of hypothalamic energy homeostasis.

1.5.4. Neurotransmitters and fat metabolism: 'The brain-fat axis'

Whilst AT stores have to match the body's overall surplus or deficit of energy there must input about the body's energy status from one or more sources. Nerve fibres are located in close proximity of adipocytes (Cinti, 2001). It is well established that various neurotransmitters especially neurotransmitters of the sympathetic system can influence AT. These can be activated by stimulation of the autonomic nervous system via NTS and allow feedback on energy status to the AT as shown by the example of central acting leptin (see below). The neurotransmitter and gut hormone ghrelin which will also be further discussed in a separate section can also influence adipose tissue mass through central activation as shown by intracerebroventricular (*icv*) infusion (Theander-Carrillo, 2006). Whilst it may appear surprising to find ghrelin as the orphan ligand of the growth hormone secretagogue receptor (GHSR) predominantly produced in the gastrointestinal tract and act as neurotransmitter in the brain (Kojima, 1999), it is reasonable to assume that neurotransmitters including ghrelin may also be

secreted by adipocytes. Some neurotransmitters have more recently been shown to be present in WAT at mRNA level (Yang, 2003).

	Mode of CSF entry	CSF levels and obesity	Hypothalamic action	Receptor knock out
Insulin	Saturable entry (Baura, 1993)	RIA: no evidence of presence in human CSF with fasting; higher in obese Zucker rats (Stein, 1983)	Decreases food intake (Bruning, 2000) through action at ARC	NIRCO mice- obese (Bruning, 2000)
Leptin	Saturable (Caro, 1996)	About 100 times lower than serum levels (Caro, 1996)	Decreases food intake with signalling at ARC (Schwartz,1996)	Ob/ob and db/db (receptor mutation) mice- very obese (Campfield, 1995; Chen, 1996)
NPY	Entry by diffusion (Kastin, 1999)	140pg/ml, about 2 times higher than serum levels, decrease with weight loss (Nam, 2001)	Orexigenic hormone, feeding effect via Y1,4,5 (Wynne, 2005)	NPY null mice are normal (Palmiter, 1998), reduced food intake or weight gain with Y1 and Y2 knockout.
PYY	Non-saturable entry (Nonaka, 2003)	Increased in bulimia (Berrettini, 1988), no BMI correlation reported	Anorexic via Y2 at ARC (Batterham, 2002)	Failure of PYY_{3-36} to decrease food intake in Y2 null mice (Batterham, 2002)
GLP-1	Non- saturable (Kastin, 2002)	NA	Anabolic action via CRH neurons in PVN and SN (Larsen, 1997)	GLP-1R knock out: lean mice, normal food intake (Scrocchi, 1996)
Ghrelin	Saturable entry (Banks, 2002)	Negatively correlated with obesity, levels of 14 -20 pg/ml (Tritos, 2003)	Anabolic action via GHR-1a in ARC (Tschop, 2002)	Normal weight and food intake in ghrelin and GHR-1 null mice (Sun,2004)
Adiponectin	NA	Present in mice (Qi, 2004); in rats (Caja, 2005b), not found in human CSF (Spranger, 2006)	Decrease of body weight through action in PVN (Qi, 2004)	Hypertension, impaired insulin sensitivity and fatty acid clearance (Kubota, 2002)
Resistin	NA	Not found in rats (Caja, 2005a), no other reports	*Icv* administration promotes short term satiety (Tovar, 2005);	Low blood glucose (Banerjee, 2004); treatment of knockout mice with resistin does not change body weight or food intake (Rajala, 2003)

Table 1.5.3.: Entry and central effect of adipokines and proteins involved in hypothalamic energy homeostasis summarised from various sources as referenced. NIRCO: neuron specific insulin receptor knockout. NA: not available.

1.6. Leptin

1.6.1. Leptin in adipose tissue

Leptin, or OB protein, is a 16kDA protein consisting of 167 amino acids. It was identified through cloning of the gene responsible for severe obesity of the *ob/ob* mouse (leptin deficient mouse) in 1994; it is released predominantly from adipocytes (Zhang, 1994). Increased deposition of TG in adipose tissue raises leptin secretion. Leptin shows depot specific expression which is 2.5 times higher in Sc abdominal versus omental fat (Montague, 1997). Its peripheral actions ranges from activation of the immune system, haematopoesis and angiogenesis; it is also a modulator of fertility and can act as growth factor in the brain (Rayner, 2001). Moreover, it is an important regulator of energy homeostasis especially through central regulation of satiety which will be discussed separately in Section 1.5., but also a regulator of peripheral metabolism. Circulating leptin levels show strong positive correlation with fat mass (Considine, 1996) that has led leptin to be regarded as 'the lipostatic factor' (Havel, 2004). Complete leptin deficiency in rodents and humans as well as defects in the leptin receptor result in marked hyperphagia and severe obesity (Pelleymounter, 1995; Montague, 1997; Strobel, 1998), which is reversible with recombinant (rh) leptin treatment through reduction of hyperphagia and fat mass (Farooqi, 1999). These mutations are however rare and leptin plasma levels in ordinary human obesity are severely elevated (Considine, 1996), which is thought to be due to leptin resistance. Thus, not surprisingly, leptin treatment of obese patients without leptin deficiency does not result in effective weight reduction (Hukshorn, 2000). Leptin deficiency and gene mutations of leptin and its receptor carry the clinical phenotype of obesity. In leptin deficiency or

resistance excess TGs are stored in maladaptive non-adipose tissue highlighting the potential of leptin as protector from ectopic lipid storage. This protective effect is thought to be mediated through upregulation of AMPK (Minokoshi, 2002; Unger, 2003). Leptin is antidiabetogenic: it lowers glucose and improves insulin signalling (Harris, 1998; Minokoshi, 2004), but leptin can also inhibit insulin gene expression through use of other receptor isoforms and inhibit insulin secretion in the pancreatic β-cells (Ahren, 1999; Seufert, 1999; Kieffer, 1997). Leptin is tightly regulated by SNS innervation of WAT (Rayner, 2001), which could be responsible for reduced leptin with fasting and cold exposure. Other important regulators of leptin in WAT are glucocorticoids (Halleux, 1998), cytokines like TNF-α and insulin (Rayner, 2001; Cammisotto, 2005) (Figure.1.6.1.). There is also evidence that nutrients like glucose and lipids induce leptin mRNA expression (Wang, 1998).

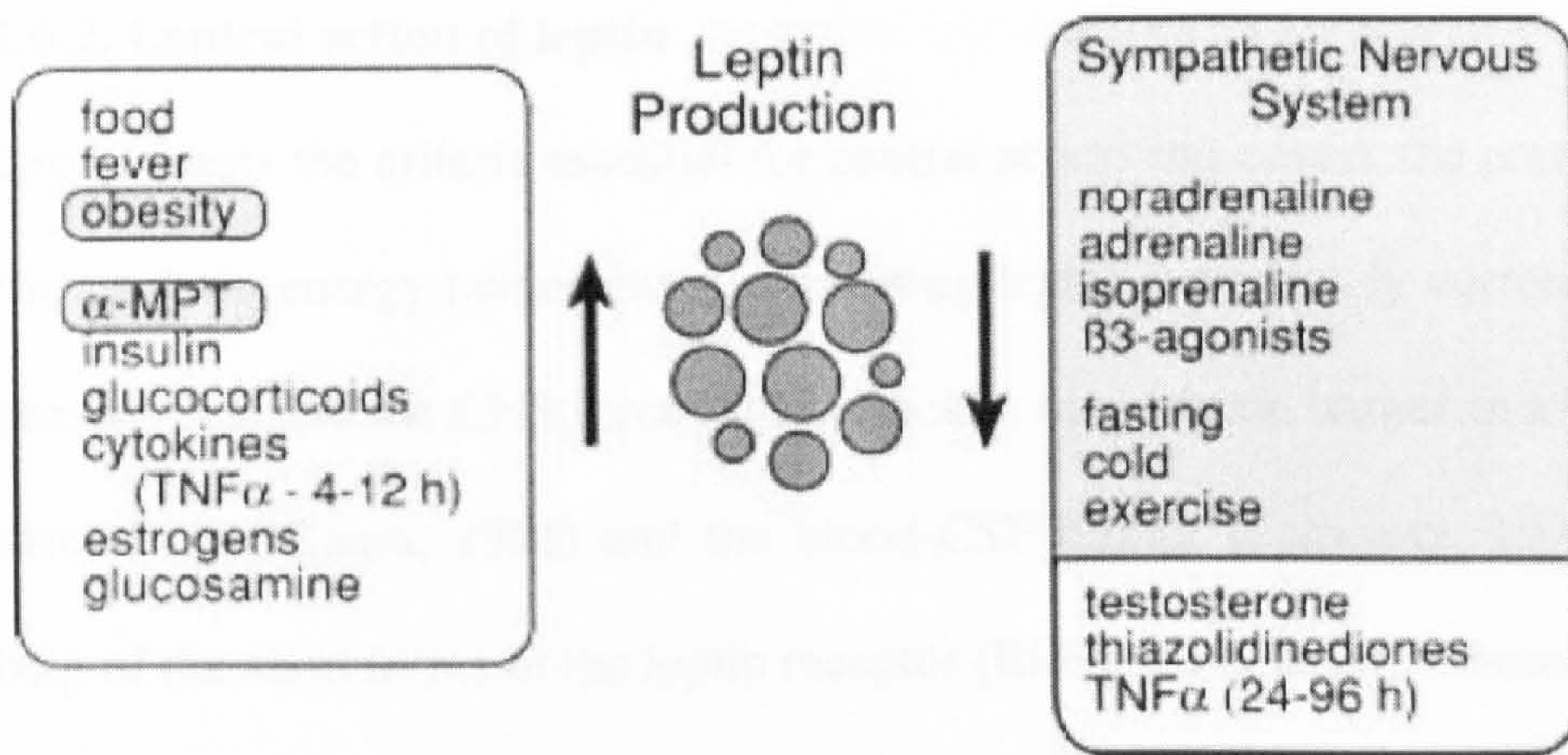


Figure 1.6.1: Regulators of leptin production in WAT. Factors which involve the SNS are shaded (Adapted from Rayner 2001).

Leptin follows a circadian rhythm and rapid changes in ob-gene expression, *e.g.* with fasting are rapidly reversible and not associated with a change in body adiposity (Trayhurn 1995). Thus a short term leptin regulation needs to be

carefully distinguished from a long term regulation which can enable energy storage.

The leptin receptor is a member of the cytokine receptor family. Alternative splicing of the gene product leads to different transcripts: the long, three short and the soluble forms of the leptin receptor (Chan, 2002). The soluble receptor is the major leptin binding protein and modulates leptin action by increasing its clearance and preventing binding to the ubiquitous membrane receptor (Lammert, 2001; Huang, 2001). The relationship of free and bound leptin levels is functionally important and altered with BMI and age (Magni, 2005). Leptin is able to interact with various signalling factors and establish cross-talk with different signal transduction systems, *e.g.* it can signal with the Jak-Stat and IRS-P13K pathways (Fruhbeck, 2006) as described above.

1.6.2. Central action of leptin

Leptin meets the criteria essential for central action and covers the prerequisites for a role in energy homeostasis: circulating leptin is positively correlated with obesity; it enters the CNS through the vascular blood-brain barrier in a saturable mechanism (Baura, 1996) and the blood-CSF barrier (Schwartz, 1996b) with help of the short forms of the leptin receptor (El-Hashimi, 2000). Choroid plexus binding sites for leptin have been confirmed by Malik (1996). Leptin inhibits NPY mRNA expression/ synthesis and NPY release in the ARC and the leptin receptor ob-R is co-expressed with NPY in the neurons of the ARC (Stephens, 1995; Schwartz, 1996a). In rodent models of leptin deficiency (ob/ob mouse) NPY is over-expressed consistent with a negative feedback regulation (Wilding, 1993). Simultaneously to NPY inhibition leptin elicits opposite activity on the

anorexigenic POMC neurons which contains ob-R and increases its expression in the ARC (Thornton, 1997). αMSH (melanin stimulating hormone) derived from the precursor POMC is also anorexigenic and acts through the melanin concentrating hormone receptor MC4-R in the LHA, which is another downstream regulator of leptin. αMSH can also be found in the general circulation and inhibits leptin expression in the adipocyte through MC1R and MC5R (Shimizu, 2005), which may be part of a feedback mechanism. CSF leptin levels are higher in the obese than in lean subjects and decrease after weight loss (Nam, 2001); and serum leptin levels have a similar association with BMI. The fact that leptin signalling at the hypothalamus inhibits food intake but the plasma and CSF levels are elevated in obesity led to the theory of leptin resistance in obesity. Interruption of hypothalamic pathways, *e.g.* through microinjection of colchicine inhibit the effect of leptin which implies the importance of intact central pathways and also suggests the possibility of central leptin resistance in obesity (Kalra, 1998). Additionally, BBB studies propose that a considerable part of the leptin resistance is due to a defect in the transport system which may be further enhanced by obesity related hypertriglyceridaemia (Caro, 1996; Banks, 2003). This disruption could potentially be bypassed by intranasal leptin administration (Kastin, 2006). Another proposed mechanism of leptin resistance is disturbed leptin signalling at the level of the hypothalamus (Fei, 1997). Neuronal activation of the leptin receptor involves the Jak-Stat pathway (Cowley, 2001) and SOCS-3 expression (see also Section 1.2.7.) and its disturbance may lead to resistance (Gao, 2004). A reduction of the intrahypothalamic leptin signals leads not only to increased hunger, but also to suppression of plasma thyroid hormone levels (Spiegelman, 2001) which are

otherwise increased by leptin (Ahima, 1996). Taken together, the precise mechanisms underlying leptin resistance have not yet been securely established, but emphasise the central role of the adipokine leptin in appetite control.

1.6.3. The feedback cycle of leptin

Leptin exhibits feedback via sympathetic neurons to WAT. Evidence of sympathetic connection between appetite regulating centres and AT was gained through studies using retrograde tracers and injection of pseudorabies virus (PRV) into fat pads, in hamsters. These showed that PRV could be traced back to the NST of the brainstem (Mercer, 1998) and the PVN (Bartness, 1998). Stimulation of the PVN, which amongst other regions of the hypothalamus contains leptin receptors, induces sympathetically mediated lipolysis (Bartness, 1998) which in theory should lead to fat shrinkage in return.

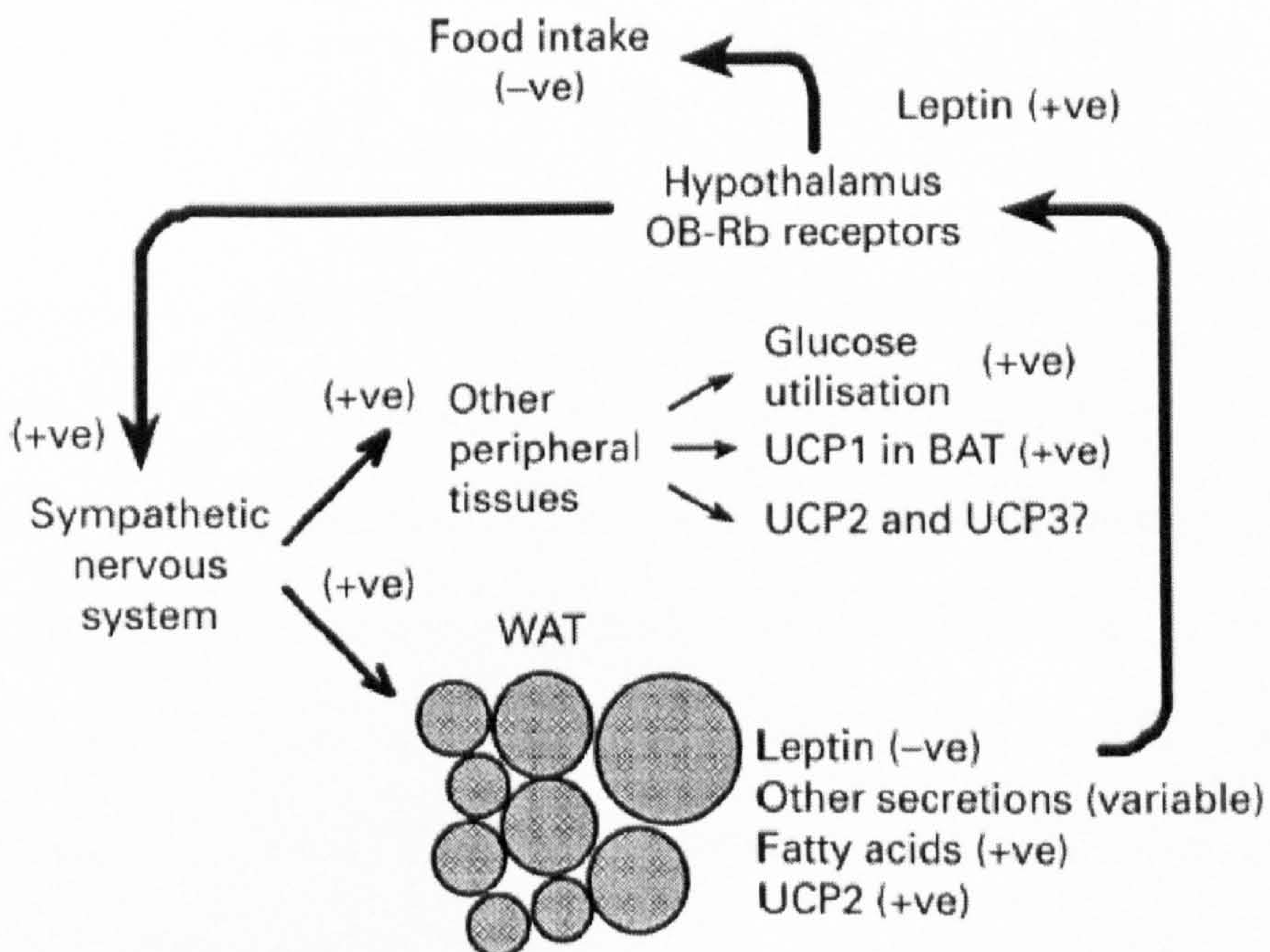


Figure 1.6.3.: Feedback loop between fat and brain via SNS. Hypothalamic leptin signalling not only inhibits food intake but also stimulates sympathetic activity through NA release. Sympathetic stimulation increases glucose utilisation, and possibly also heat production through uncoupling proteins in fat and muscle and provides negative feedback to adipocytes to limit further leptin production (Adapted from Rayner, 2001).

Further peripheral studies have identified a short feedback loop in the adipocyte itself, by which leptin's paracrine function is inhibited by leptin itself via post receptor blockade by SOCS-3 (Wang, 2005). This enables fat storage when AT-derived leptin levels are rising, *e.g.* with increasing fat mass and would otherwise not succeed in obesity and enhance ectopic fat accumulation.

1.7. Adiponectin

1.7.1. Adiponectin in adipose tissue and circulation

Adiponectin also known as ACRP30 (30kDa protein), AdipoQ and GBP28, was discovered in 1993 and is thought to be one of the most abundant adipocyte-specific gene products (Trujillo, 2005; Kadowaki, 2005).

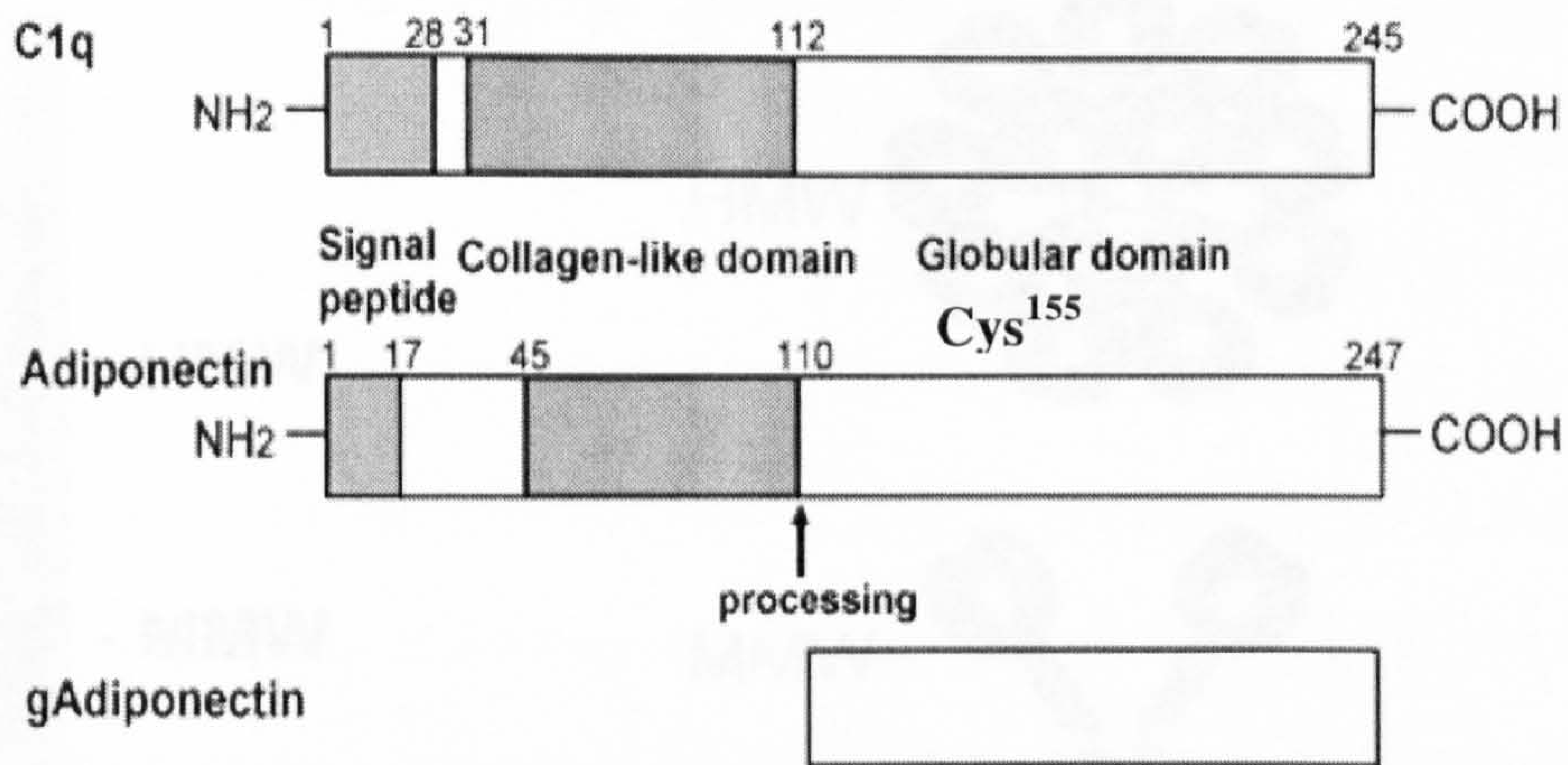


Figure 1.7.1.1.: Structure and domains of adiponectin. Adiponectin is composed of an N-terminal collagen-like sequence and a C-terminal globular region. C1q is shown in comparison. gAdiponectin is the truncated form of adiponectin (Adapted from Kadowaki, 2005).

Adiponectin belongs structurally to the collagen complement C1q family and is composed of a distinct globular head domain and collagen-like tail (Figure 1.7.1.1.). The adipocyte produced hormone adiponectin is abundant in serum and circulates in multimers held together with disulfide bonds, which range from trimers to high molecular weight forms (HMW) of 12-18 subunits (Pajvani, 2003) (Figure 1.7.1.2.). The cysteine residue at position 155 is critical to HMW formation and integrity of its structure (Waki, 2003). Another circulating form of adiponectin is the truncated form of a globular domain only (Kadowaki, 2005). However, the full length forms are the main forms in human plasma with metabolic activities different from the truncated form (Spranger,

2006). The C-terminal domain of the trimeric forms of adiponectin activate AMPK and lead to increased fatty acid oxidation and reduction of serum glucose (Yamauchi, 2002; Berg, 2001). The hexameric, medium molecular weight (MMW) forms and HMW multimers of adiponectin, however, activate NF-κB pathways and inhibit cell apoptosis (Tsao, 2003; Hug 2004).

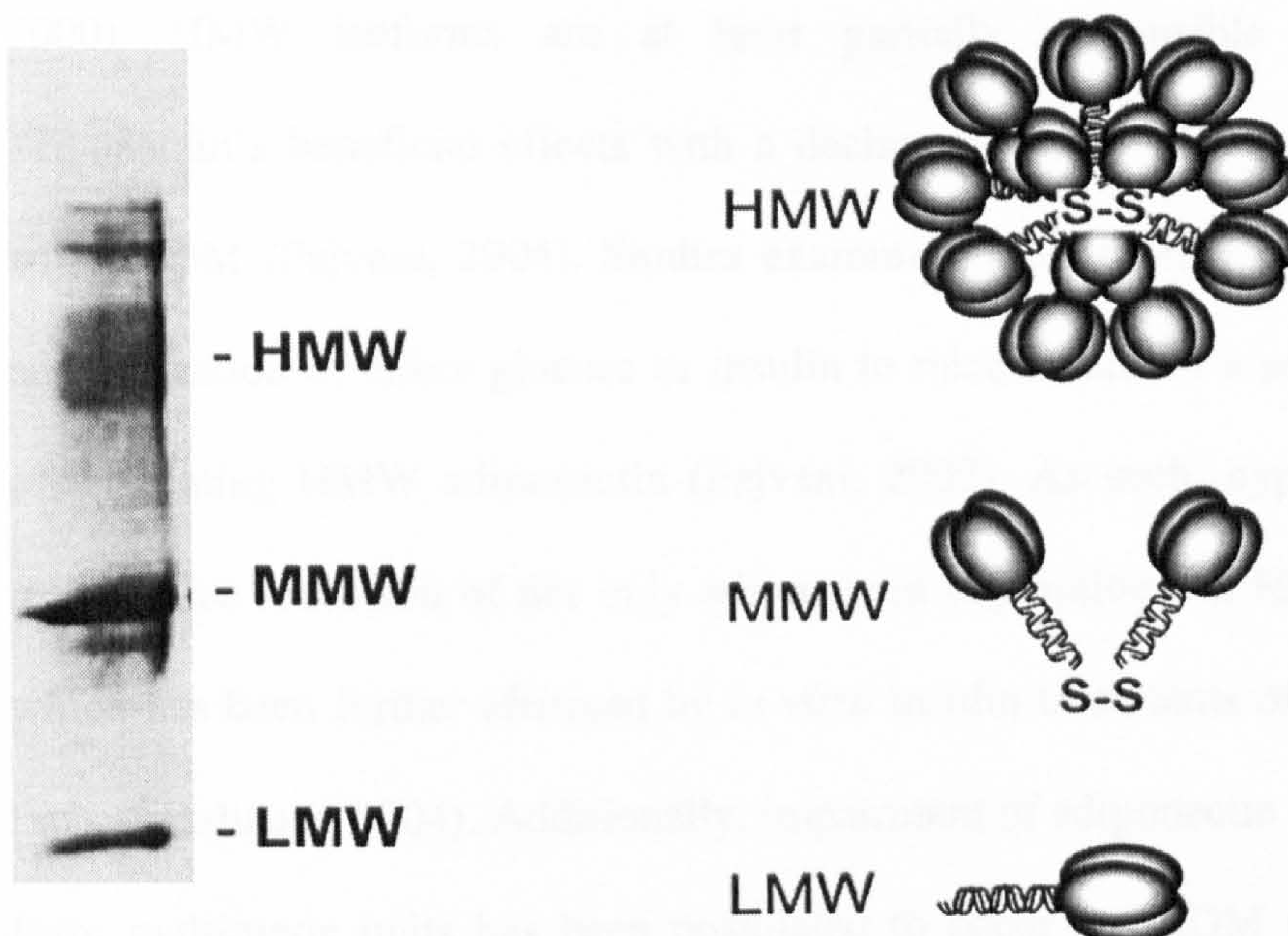


Figure 1.7.1.2.: Multimer formation of adiponectin. SDS PAGE gel on left depicting three different isoforms. MMW, Medium molecular weight; LMW, low molecular weight; S–S, disulfide bridge (Kadowaki, 2005).

Adiponectin inhibits adipocyte differentiation (Yokota, 2002) and is a key regulator of insulin sensitivity and tissue inflammation and has anti-arteriosclerotic effect. Circulating adiponectin levels are reduced in T2DM, obesity (Hotta, 2001; Arita, 1999) and obesity related hypertension (Ohashi, 2006) as well as cardiovascular disease (Arita, 1999; Okamoto, 2000). Adiponectin serum levels are in fact predictive of T2DM (Lindsay, 2002) and cardiovascular disease (Pischon, 2004) and high levels are associated with smaller MI risk in men, respectively. But, adiponectin levels are not predictive of future BMI (Yamamoto, 2004). The adipocyte secretion rate of adiponectin in

obesity declines by about 30% per weight unit (Hoffstedt, 2004), which is able to override the increased adiponectin production resulting from an increase in adipocytes/ fat mass with a net result of decreased circulating adiponectin levels in obesity. The human adiponectin gene at genomic locus 3q27 is thought to be linked with susceptibility to T2DM and the metabolic syndrome (Kissebah, 2000). HMW isoforms are at least partially responsible for some of adiponectin's beneficial effects with a decline of HMW structures in subjects with T2DM (Pajvani, 2004). Studies examining the isoforms have shown that administration of either glucose or insulin to mice results in a severe reduction of circulating HMW adiponectin (Pajvani, 2003). As such, hyperinsulinaemia may induce reduction of not only adiponectin expression but HMW formation which has been further affirmed by *in vitro* insulin treatments of adipocyte cell lines (Fasshauer, 2004). Additionally, impairment of adiponectin in the ability to form multimeric units has been postulated to occur in T2DM patients (Waki, 2003).

The globular head domain appears to enhance AMPK activity in myocytes, for which it has high affinity, and reduces muscle triglyceride and NEFA content through an increase of ß-oxidation (Yamauchi, 2002). The full length adiponectin enhances AMPK in the liver, where it inhibits the expression of hepatic gluconeogenesis and the rate of endogenous glucose production in mice (Combs; 2001) which ultimately reduces hepatic glucose output (Berg, 2001; Yamauchi, 2002). The expression of adiponectin is not only reduced by insulin, but also IL-6 and TNF-α (Kappes; 2000) and increased by PPAR-γ agonists in form of pharmacologically available insulin sensitising drugs known as thiazolidinediones (TZDs), *e.g.* Rosiglitazone (RSG) (Combs, 2002).

In search of receptors mediating the antidiabetic action of adiponectin, two adiponectin receptors were cloned distinct from G-protein coupled receptors termed adiponectin receptor 1 (AdipoR1) and 2 (AdipoR2) which share 66% sequence homology (Kadowaki, 2006). They were shown to mediate many of the metabolic effects of the globular and full-length protein, *e.g.* increased AMPK, PPR-α activities, glucose uptake and fatty-acid oxidation (Yamauchi, 2003; Kadowaki, 2005). Both receptors are expressed in adipocytes whilst AdipoR1 is predominantly expressed in the skeletal muscle and AdipoR2 is abundant in the liver. These receptors appear downregulated in obesity (Kadowaki, 2005) and are negatively regulated by insulin (Tsuchida, 2005). A novel extracellular receptor, T-cadherin, which is expressed in endothelium and smooth muscle, has been observed to bind the more abundant larger multimeric forms of adiponectin (Hug, 2004) and its function needs yet to be further clarified.

1.7.2. Adiponectin in the brain

More recent studies suggest central action of adiponectin in rodents and a role in thermoregulation. Rodent studies examining adiponectin's effect by use of *icv* injection of adiponectin observed weight loss and increased cellular Fos nuclear protooncogene (c-Fos) activity (immediate early gene and a marker of early neuronal stimulation (Hughes, 1995) in the PVN of wildtype mice (Qi, 2004). However, a more recent study in humans failed to show presence of adiponectin in CSF (Spranger, 2006) and it remains unclear whether adiponectin has a central role in humans.

1.8. Resistin

1.8.1. Resistin in adipose tissue

Resistin or FIZZ3 is a 14kDa polypeptide; it belongs to a family of cysteine-rich-C-terminal proteins, known as resistin like molecules (RELM, RELMalpha/FIZZ1 and RELMbeta/FIZZ2) of FIZZ (found in the inflammatory zone) involved in inflammatory processes (Holcomb, 2000; Steppan, 2001b). Resistin was thought to be secreted and circulate as dimer (Banerjee, 2001), but more recent evidence shows that it circulates as a multimer of a dimeric form dependent upon a single intermolecular disulfide bond (Graveleau, 2005). The complex structure of resistin is shown in Figure 1.8.1.; it comprises a carboxy-terminal disulfide-rich beta-sandwich "head" domain and an amino-terminal alpha-helical tail segment. The disulfide bridges enable formation of a hexamer and trimer which may dictate the bioactivity of this molecule (Patel, 2004).

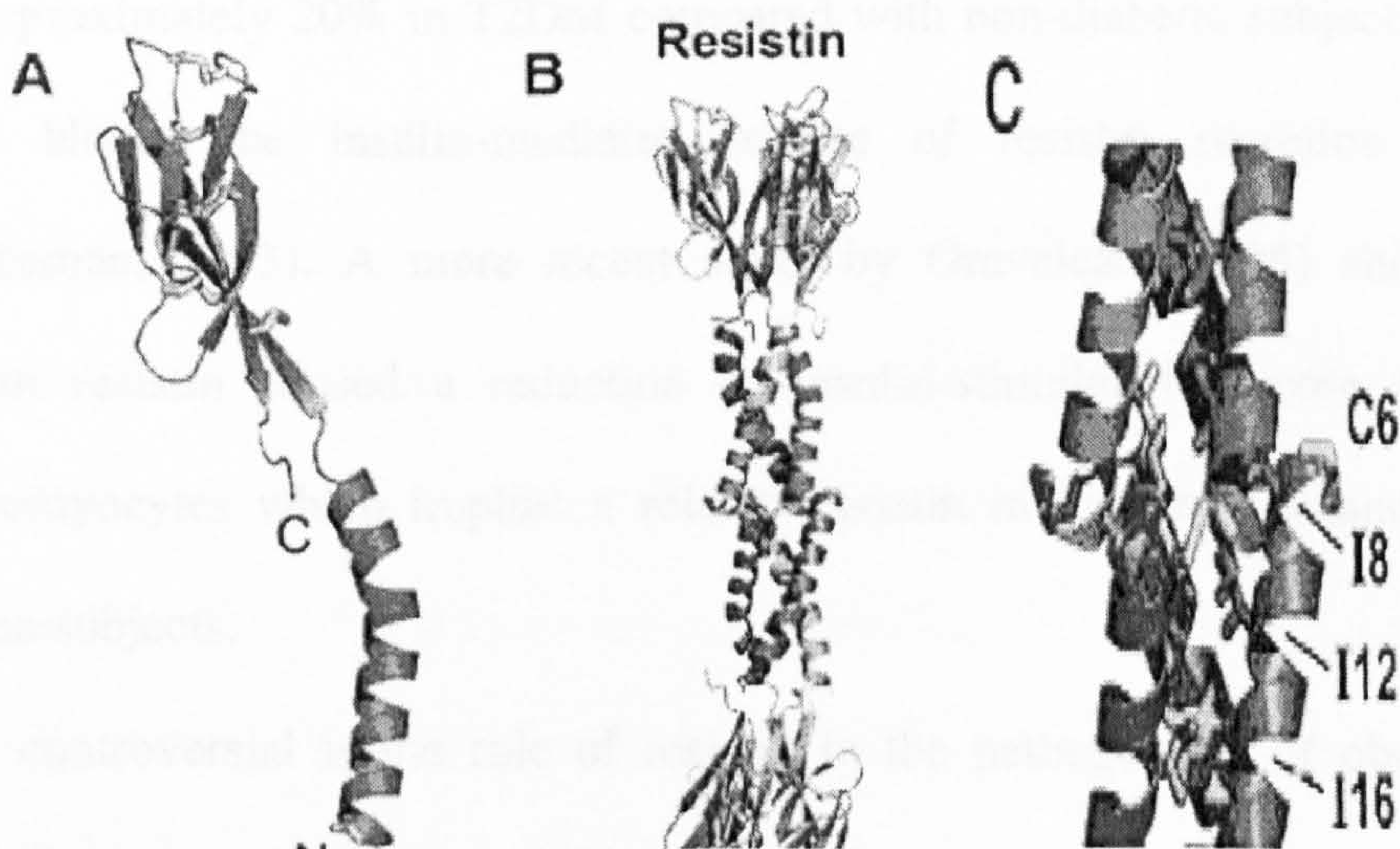


Figure 1.8.1.: X-ray crystallography of resistin molecule. A: Resistin monomer. B: Resistin hexamer. C: Orientation of disulphide bridges binding n-terminal regions (Adapted from Patel, 2004).

The resistin gene was discovered in the search of genes which are downregulated by PPAR-γ agonists (Steppan, 2001a). Injection of recombinant

resistin in mice has shown to induce insulin resistance. This effect was neutralized by antibodies to resistin leading to normalization of glucose homeostasis. In addition, resistin also inhibits adipogenesis in murine 3T3-L1 cells (Steppan, 2001a). Taken together, these studies support a role of resistin in the pathogenesis of diabetes and obesity (Steppan 2001b, Kim 2001). The mechanism of resistin action is thought to be an increase of hepatic glucose production and glucose uptake and impairment of insulin signalling through mechanisms which remain to be identified. Resistin's effect on insulin metabolism in humans is more controversial and some studies have failed to show a correlation with insulin sensitivity (Savage, 2001) with conflicting results of resistin expression following TZD treatment (Way, 2001; Patel, 2003) and the regulation of resistin by insulin, *e.g.* on cultured human adipocytes (Way, 2001; Rajala 2002). Our group has shown that serum resistin is increased by approximately 20% in T2DM compared with non-diabetic subjects and that RSG blocks the insulin-mediated release of resistin secretion in vitro (McTernan, 2003). A more recent study by Graveleau (2005) showed that human resistin caused a reduction in insulin-stimulated glucose uptake in cardiomyocytes which implies a role of resistin in insulin resistance also in human subjects.

Also controversial is the role of resistin in the pathogenesis of obesity with contradicting associations with body mass (Tovar, 2005), which may in part be explained by the counter-regulation of protein and its mRNA expression (Rajala, 2004). The net effect of increased adipocyte number in obesity on resulting serum resistin levels may be overshadowed by resistin's depot specific distribution which is best observed in central obesity. Whilst there is some data

showing resistin mRNA levels are highest in human and rodent visceral fat (Atzmon, 2002), data from our study group suggests that there is no significant difference amongst abdominal Sc and omental depot protein expression of resistin (McTernan, 2002) and showed that the expression is about 4-fold higher in abdominal AT *versus* thigh AT (McTernan, 2002). The source of resistin in human visceral fat is not solely the adipocyte, but a mixture of cells from preadipocyte, epithelial cell, and macrophage, which may increasingly infiltrate adipose tissue in obesity and may explain the association with insulin resistance (Fain, 2004). Furthermore, resistin is an inflammatory marker and serum resistin levels are associated with high serum CRP (Al-Daghri, 2005). In agreement with this, our group showed that resistin influences proinflammatory cytokine levels in isolated Sc adipocytes as well as pathways of the innate immunsystem (Kusminski, 2007).

1.8.2. Resistin and central action

Resistin mRNA expression and immunoreactivity has been noted in the ARC and other areas of the mouse hypothalamus (Morash, 2002; Wilkinson 2005). Evidence from neurophysiological studies shows that resistin inhibits the stimulated release of dopamine and noradrenaline from hypothalamic neuronal endings similar to leptin (Brunnetti 2004) and central administration of resistin was shown to promote short term satiety in rats (Tovar, 2005). However, resistin was not found in rodent CSF and there is no current evidence of its ability to cross the BBB (Caja, 2005a). Also, to date, there have been no reports which examined the central role of resistin in humans.

1.9. The pancreatic polypeptide family and DPP-IV

1.9.1. Neuropeptide Y and the brain

NPY is a member of the pancreatic polypeptide (PP) hormone family and is related to the pancreatic polypeptide Y (PPY) and PYY. NPY is one of the most ample neurotransmitters in the brain and is one of the most potent orexigenic hormones. It was first isolated in 1982 (Tatemoto, 1982) and is derived from splicing of the precursors prepro-neuropeptide Y in form of a 36 amino acid peptide (Figure 1.9.1.1.).

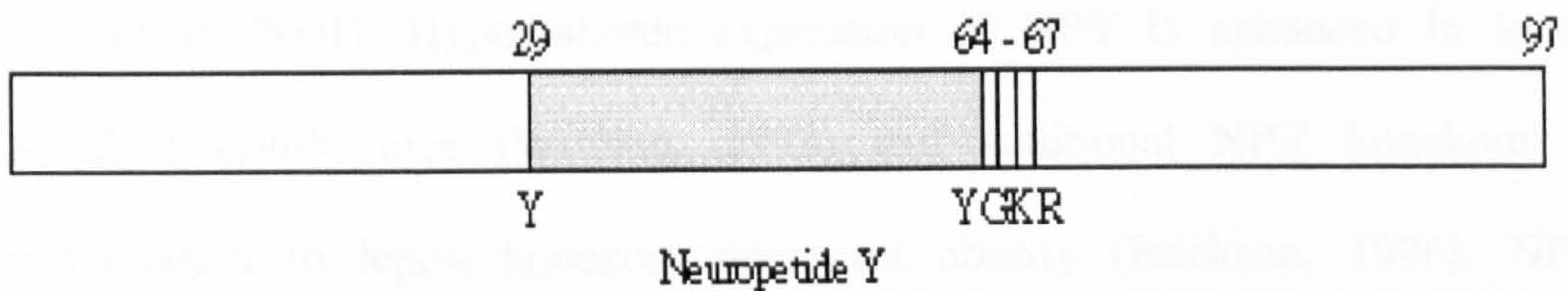


Figure 1.9.1.1.: Structure of human prepro-neuropeptide Y. The protein NPY is shown in grey (Minth, 1984).

NPY binds to transmembrane-domain G-protein coupled receptors, NPY Y1 – Y6 (Larhammar, 1996), of which Y3 has not been cloned yet (Zukowska, 2003) and Y6 is not functional in species other than the mouse (Herzog, 1992); as such there are 4 human recognised receptors: Y_1, Y_2, Y_4 and Y_5. This receptor family lacks sequence identity, particularly between Y_1, Y_2 and Y_5 receptors, which are only 30% identical (Brain, 2006). The activation of these receptors modifies the action of adenyl cylclase and leads to changes of intracellular calcium (Toth, 1993).

NPY is a neurotransmitter abundant in the brain, peripheral sympathetic nerves and adrenal medulla, where it is colocalized with catecholamines but is also found in most other tissues (Everitt, 1984). In the CNS, NPY is an inhibitory neurotransmitter and exhibits anxiolytic, anti-stress, anti-depressant, anti-

convulsant and anti-nociceptive actions in addition to its hypertensive, potent appetite-stimulating effects and has potential to shift circadian rhythms. NPY is also involved in the regulation of angiogenesis, vasoconstriction, mood regulation and fertility (Chronwall, 2004). Microinjection of NPY into the paraventricular nucleus of the hypothalamus (PVN) stimulates feeding (Stanley, 1986) and chronic *icv* infusion causes obesity (Clark, 1984). ARC NPY secretion and expression are negatively regulated by leptin and insulin (Stephens, 1995; Schwartz, 1996b; Sahu, 1995) and positively by ghrelin (Nakazato, 2001). Hypothalamic expression of NPY is enhanced in leptin deficient *ob/ob* mice (Wilding, 1993) and additional NPY knockout in combination to leptin knockout decreases obesity (Erickson, 1996). NPY circulates in serum and CSF. Whilst a clear association with serum levels and BMI could not be established (Baranowska, 2000), it appears that CSF NPY levels do not correlate with BMI, but decrease after weight loss (Nam, 2001).

The effect of NPY on energy balance is mediated by a combination of receptors and Y1, Y2, Y4 and Y5 have been postulated to be responsible for the appetite regulation of NPY (Wynne, 2005). Whilst knockout mice lacking NPY from central and enteric neurones are normal weight with normal response to starvation (Erickson, 1996), Y1 knockout mice show transiently reduced daily food intake and higher leptin levels (Pedrazzini, 1998) and Y2 hypothalamus specific knockout mice show reduced body weight and increased expression of hypothalamic NPY (Sainsbury, 2002). This observations led to the study of NPY receptor agonists and antagonist in the hope to reveal potential new anti-obesity agents. This has proven difficult because the multiple effects mediated by these receptors besides weight regulation, *e.g.* Y4 receptor knockout mice

show overtly aggressive behaviour. Whilst Y5 antagonists failed to prove an antiobesity effect in clinical trials (Block, 2002), studies are currently evaluating the prospects for Y2 agonists. Y2 receptors have a predominantly presynaptic location where they act to inhibit NPY release. Y2 also mediates anticonvulsant actions and antinociceptive effects of NPY in the hippocampus making Y2 a potential anticonvulsant therapeutic (El-Bahh, 2005) and analgesic, *e.g.* for the treatment of neuropathic pain (Moran, 2004).

1.9.2. NPY and the adipocyte

NPY is coreleased with noradrenaline, it favours energy storage by central action on appetite but central administration also increases LPL activity in WAT suggesting a peripheral role of NPY in lipogenesis (Billington, 1991; Zarjevski, 1993, Karvonen, 1998). NPY mediates an antilipolytic effect in human adipose tissue (Valet, 1990; Castan, 1994; Labelle, 1997) which is thought to be mediated by receptor Y1 in humans (Bradley, 2005), the same receptor may also stimulate leptin secretion in adipocytes (Serradeil-La Gal, 2000) whilst the role of Y5, which also shows gene expression in adipose tissue, is not clear. Experiments with *in vitro* co-culture of rat sympathetic neurons with the 3T3-L1 cell line of adipocytes (Turtzo, 2001) show inhibition of ß-adrenergic lipolysis. This inhibition was suggested to be the effect of NPY induction via the sympathetic system (Bartness, 2005). Cell treatment with NPY in co-culture was also shown to inhibit leptin secretion (Bartness, 2005) where as *iv* injection of NPY increased leptin mRNA and Y1 mRNA expression in sheep adipocytes (Dyer, 1997).

1.9.3. PYY

The gut hormone PYY belongs to the same family as NPY with which it shares 70% of sequence homology. PYY is released postprandially in two forms: PYY_{1-36} and PYY_{3-36} and its levels are proportional to meal size (Adrian, 1985). Like NPY, PYY is also converted by DPP-IV (Eberlein, 1989). PYY signals through NPY receptors. PYY_{1-36} is an agonist at the Y1/Y2 receptors and promotes feeding, whereas PYY_{3-36} is an antagonist at the Y2 receptor and inhibits feeding (Grandt, 1994), an effect which is absent in Y2 knockout mice (Batterham, 2002). PYY_{3-36} can cross the BBB via diffusion (Nonaka, 2003) and inhibits ARC NPY neurons and reduces hypothalamic NPY mRNA expression (Batterham, 2002). Intravenous administration of PYY_{3-36} reduces hunger and measured food intake by 30% in humans and its effect lasting up to 12 hours (Batterham, 2002), whilst *icv* administration of PYY_{1-36} and PYY_{3-36} increase food intake in rodents probably because of a bypass of ARC Y2 receptors and action through Y1 and Y5 (Stanley, 2005). Furthermore, a recent report has noted that the attenuated response of PYY in obese subjects is associated with reduced satiety (le Roux, 2006) and PYY_{3-36} could thus offer potentially effective antiobesity therapy.

1.9.4. DPP-IV

The presence of dipeptidyl peptidase-IV (DPP-IV), a highly specific protease also known as CD26 in the immune system, influences amongst other proteins the affinity of NPY to its receptors by acting as a converting enzyme. DPP-IV cleaves off proline in the penultimate position of many peptides like NPY, PYY and GLP-1, but NPY_{1-36} is one of its best substrates (Mentlein, 1999). DPP-IV is

found in the endothelium and epithelial cells, serum and activated immune cells (Mentlein, 1999). It shifts NPY activities from Y1 towards receptor Y2 and Y5 pathways with generation of NPY_{3-36} from NPY_{1-36}, and can therefore also be seen as endogenous Y1 receptor antagonist (Zukowska-Grojec, 1997a) in a similar way it modulates the action of PYY.

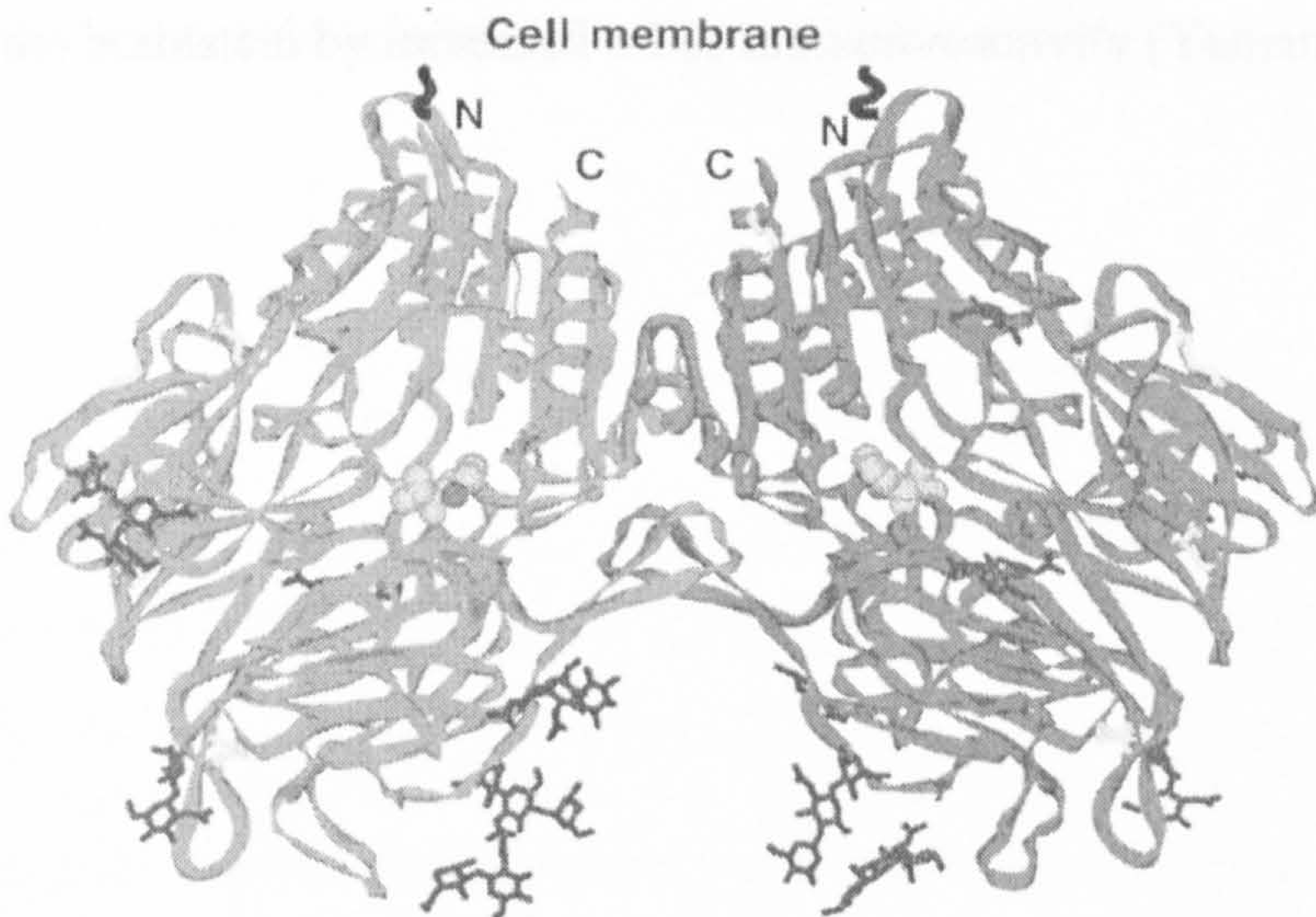


Figure 1.9.4.: Crystallographic structure of DPP-IV which consists of two subunits. The full-length DPP-IV is a type II transmembrane protein (Adapted from Rasmussen, 2003).

DPP-IV inhibitors have recently become available as anti-diabetic pharmacological agents to sustain a prolonged incretin effect (Ahren, 2004). The so called incretin effect has been revealed by the finding that after oral intake of glucose plasma insulin levels are greater than those observed when glucose is given intravenously (McIntyre, 1965) and is entertained by insulin release through secretion of gut hormones like glucagon like peptide -1 (GLP-1) through contact with food. GLP-1 is a 30 amino acid protein derived from the cleavage of the proglucagon molecule derived primarily from the gut (enteroendocrine L-cells from the terminal ileum). Whilst DPP-IV truncates the

N-terminus of the active metabolite by cleavage of 2 amino acids and is responsible for the short half-life of GLP-1 (7-36) of 1-3 minutes (Hui, 2005), the levels of immunoreactive GLP-1 have been shown to increase up to five fold by DPP–IV inhibition (Ahren, 2004). GLP-1 has also shown to have anorexic properties (Donahey, 1998; Alvarez, 2005). Its central action is primarily mediated through CRH neurons in the PVN (Larsen, 1997) and appears to activate the brainstem by increased c-Fos immunoreactivity (Yamamoto, 2003).

1.10. Ghrelin

1.10.1. Ghrelin and the brain

The neurotransmitter ghrelin, a 28 amino acid peptide is a product of splicing of its precursor prepro-ghrelin an 117aa peptide of 13.3kDa (Kojima, 1999).

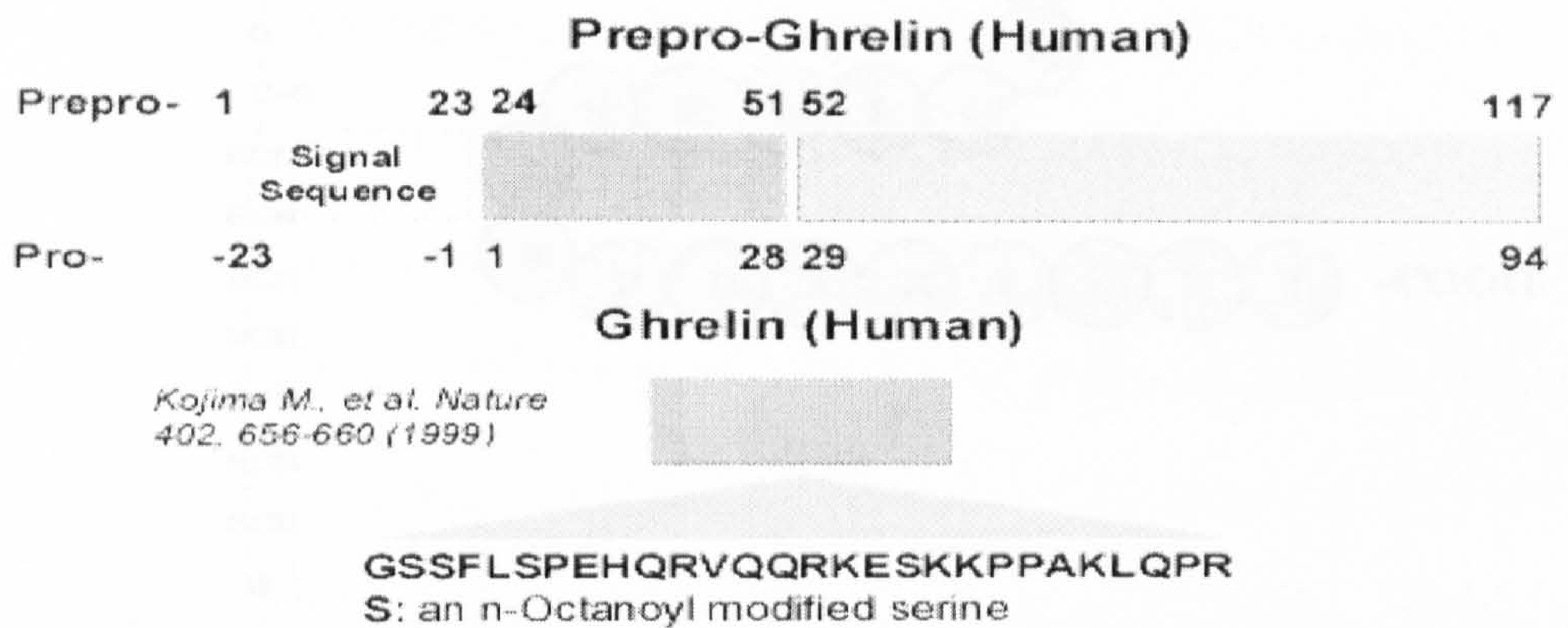


Figure 1.10.1.: Structure of Ghrelin. The molecular weight of preproghrelin is 13.3kDa and 3kDa of the mature ghrelin (Adapted from Kojima, 1999).

Ghrelin is the orphan ligand of the growth hormone secretagogue receptor type 1a (GHS-R1a), which was originally identified in the hypothalamus, but the primary production sites of this peptide are the stomach and colon (Kojima, 1999), which makes ghrelin a mediator of the gut-brain axis. Ghrelin mRNA is expressed in almost all tissues including endocrine tissues like pituitary, adrenal gland, thyroid, ovary, testis and the fat (Gnanapavan, 2002).

Ghrelin exists in two different isoforms. Des-octanoyl ghrelin does not activate GHS-R1a, which is dependent on the octanoyl modification of the third serine residue of ghrelin (Hosoda, 2000). The acetylation of ghrelin is achieved by a unique post-translational acylation with octanoic acid. The natural ghrelin acyl-modifying enzyme is not yet known. Further it is currently unclear whether des-acylated ghrelin is a precursor of acetylated ghrelin or a product of the deacylation (Kojima, 2005) and which receptor is used by des-acetylated ghrelin. The gut hormones PP and oxyntomodulin (OXM) have been shown to

suppress ghrelin hormone secretion or expression which in part explains their anorexigenic activity (Stanley, 2005).

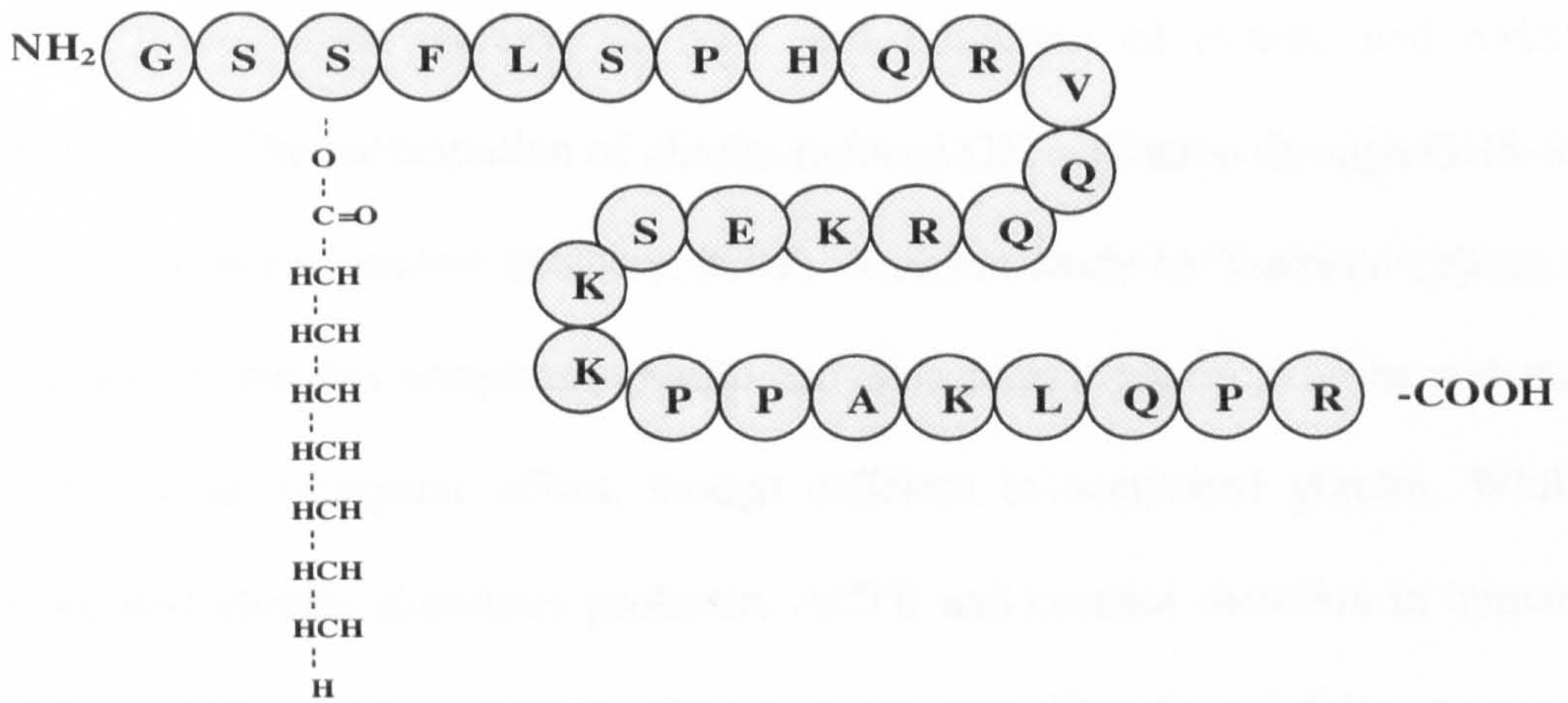


Figure 1.10.2.: Structure of human octanylated ghrelin (C8:0). Human Ghrelin is a 28–amino acid peptide in which Ser 3 is modified by a fatty acid in this case n-octanoic acid (Adapted from Kojima, 2005).

There is a strong association of ghrelin with obesity and the fasting total ghrelin plasma levels are reduced in human obesity (Tschop, 2001) and its nocturnal raise is blunted in obese subjects (Yildiz, 2004). The levels rise again after weight loss and gastric surgery (Vendrell, 2004). Ghrelin serum levels are also reduced in insulin resistance and diabetes (Poykko, 2003; Katsuki, 2004) and show an inverse relationship with circulating plasma insulin levels (Holdstock, 2003). As serum ghrelin reaches peak plasma levels just before meals and stimulated by sympathetic activation (Mundinger, 2006) after which they rapidly fall, it is thought that the role of ghrelin is to be that of a meal initiator (Cummings, 2001). Peripheral and *icv* administration of ghrelin leads to increased food intake and eventually to obesity in rodents (Tschop, 2000; Chen 2004) and also stimulates food intake in humans (Wren, 2001). The anabolic

effect is mainly mediated by GHS-R1a, through octanylated ghrelin (Kojima, 1999). Targets of the anabolic action of ghrelin are AGRP and NPY neurons located at the ARC (Tschop, 2002) via activation of GHS-R (Nakazato, 2001) which leads to an increase of NPY and inhibition of POMC and αMSH production. The participation of ghrelin induced GH activation through GHS-R1 in food intake is unclear (Stanley, 2005). A recent study by Toshinai (2006) in rats shows that des-acetylated ghrelin activates central orexin neurons and also mediates an orexigenic effect, though different to acetylated ghrelin. Whilst acetylated ghrelin stimulates prolactin, ACTH and cortisol secretion in humans (Arvat, 2001) des-acetylated ghrelin does not (Broglio, 2004). A small proportion of ghrelin is also produced centrally and ghrelin neurons are found in the hypothalamus projecting to ARC and LHA where they potentially communicated with NPY neurons and orexin neurons (Stanley, 2005). However, it is probably mainly peripheral ghrelin which mediates central effects and ghrelin has been shown to cross the BBB (Banks, 2002) with species-specific differences in transport of octanylated and des-acetylated ghrelin, *e.g.* contrary to mouse ghrelin both forms of human ghrelin can pass the mouse BBB bi-directionally (Banks, 2002). The observed differences amongst species warrant further study of ghrelin isoforms in human physiology.

1.10.2. Ghrelin and the adipocyte

Rodent studies suggest that ghrelin is antilipolytic (Morimoto, 1998). As such studies examining *icv* ghrelin have shown that it can modulate adipocyte metabolism by mRNA induction of AT regulating enzymes like LPL, which may be similar to leptin's peripheral feedback, through influence on the

sympathetic innervation of AT (Theander-Carrillo, 2002). Also, there is the possibility that ghrelin is also expressed in AT itself. Ghrelin mRNA expression was found in human AT by Gnanapavan (2002) and both ghrelin and GHR-1a have been shown to be expressed in rodent adipocytes (Zhang W, 2004) where ghrelin also induces adipocyte differentiation (Choi, 2003; Thompson, 2004). Whilst the effects of ghrelin on WAT have mainly been studied in rodents little is known on the effect of ghrelin isoforms in humans.

1.11. Aims

The adipokine leptin and its importance in central energy regulation as one of the principal regulators of cerebral catabolic action on long term energy balance not only demonstrates that 'fat communicates with the brain', but also highlights the possibility of other adipokines and hormones participating in a feedback cycle of brain-adipose tissue energy homeostasis. In addition, obesity induced leptin resistance is an example of uncertain pathophysiology. Its regulation is dependent on two important regulator of energy homeostasis – the fat and the brain - which are potential dysfunctional organs in the pathogenesis of obesity and insulin resistance. As such, the core theme of this thesis is to explore the interaction of adipose tissue and the brain to search for additional feedback circuits of energy homeostasis. The first part of the thesis will discuss the crosstalk between fat and the brain and focus on the adipokines adiponectin and resistin to determine whether these adipokines are present in CSF and whether they could have a role in human energy homeostasis. A further aim is to examine the presence of adiponectin receptors in the nuclei of the human hypothalamus, which are known to participate in energy regulation. The thesis continues with the study of the role and the presence of the neurotransmitters and the appetite regulators NPY and ghrelin in human adipose tissue. These studies will examine their impact on adipose tissue metabolism and its modulation by obesity and *in vitro* insulin treatment, which could give further insight into the pathophysiology of the metabolic syndrome. This includes exploring the interaction of these neurotransmitters with other regulators of energy homeostasis like leptin and their influence on other adipokines. Since adipose tissue innervation enables crosstalk from the brain to adipose tissue

through efferent brain stem signals as cumulative response to hypothalamic energy regulatory impulses, this thesis scrutinizes the expression of previously unidentified receptors regulating neurotransmitter release in human adipose tissue. It investigates for presence of nicotinic receptors (CHRNA) in WAT which modulate neurotransmitter release, *e.g.* NPY, and which are affected by smoking.

Chapter 2

General materials and methods

2.0. General materials and methods.

The following section contains a description of general materials and methods that were most regularly used throughout the thesis. Further information on methods specific to certain work is outlined within the appropriate chapter whilst specific reagents are documented in Appendix I and II.

2.1. Adipose tissue collection and processing

2.1.1. General

Subcutaneous abdominal, thigh and omental adipose tissue was collected from consenting subjects undergoing elective surgery, *e.g.* gastric bypass or hip surgery, also liposuction material was collected from cosmetic surgery with approval by the local ethics committee. Patients with history of malignancy, steroid treatment, endocrine therapy or acute inflammatory problems were excluded.

Adipose tissue (AT) was used fresh without use of preservatives e.g. formalin. It was chopped finely with scissors, transferred into a 50mL centrifuge tube and immediately placed in liquid nitrogen for rapid freezing. Frozen samples were transferred straight from liquid nitrogen into a -80°C freezer for storage until future use. These samples where further used for extraction of protein or RNA as is detailed in the following sections. Processing of AT continued for isolation of adipocytes and pre-adipocytes (see below).

2.1.2. Isolation of subcutaneous and omental adipocytes

Adipose tissue was finely minced with surgical scissors and removal of obvious vessels and connective tissue as much as possible and mixed with a ratio of 2:1

of collagenase (Worthington Biochemical Corporation, Lakewood, New Jersey, USA) in sterile 50mL centrifuge tubes alternatively with liposuction material: 25-30mL of liposuction material was poured into each centrifuge tubes and 10mL of collagenase added. Collagenase was prepared at a concentration of 1mg/mL reconstituted in 1x Hanks balanced salt solution (HBSS, Gibco^{BRL}, Life Technologies, Paisley, UK). The mixtures were shaken and immersed into a shaking warm water bath (37°C, 100cycles/min) for approximately 30 minutes (min), with additional hand shaking at 10 min intervals. When the mixture was very smooth and homogenous, it was filtered through sterile cotton mesh (Medistore, UK) into falcons and then centrifuged at 360 x g (g-force= 1.12 x 10^{-5} tip Radius in cm x rpm^2; rpm= revolutions per minute) for 5 min. This separates adipocytes from pre-adipocytes. The oily supernatant was discarded. The adipocyte layer (after oil layer) was poured into falcons and washed with 10 -15mL of warm (water bath) DMEM/F-12 phenol red free medium (15% foetal calf serum (FCS) and Dulbecco's minimal essential medium, both from Gibco^{BRL}, (Life Technologies™, Paisley, UK) containing 1% transferrin (Sigma, Dorset, UK), penicillin (100units/mL) and streptomycin (100µg/mL) (Sigma, Dorset, UK) and dispersed with gentle rocking. The remaining layer of previously centrifuged material contained a preadipocyte pellet and was treated separately (see below). The adipocyte Phenol red mixture was centrifuged at 190 x g for 30 seconds (s) and the wash repeated till the liquid below the densely packed layer was clear. This liquid was then removed with a 2mL pipette. The remaining dense layer of adipocytes was used for tissue culture at amounts of 1mL of adipocytes which were added to falcons with 5mL of cell culture medium to be incubated for 48 hours (hr) with the treatment of choice.

Some of the adipocytes (200 μL) were treated with 500mL of RIPA buffer for protein extraction (see below) or treated with RNALater™ (Ambion, AMS Biotech, UK) and then frozen at -80°C.

2.1.3. Preadipocyte separation

After the adipocyte layer was poured off, the remaining supernatant was carefully removed to leave a preadipocyte pellet. This pellet was resuspended in lysis buffer (see Appendix I) and left at room temperature for 15-20 min and then centrifuged at 360 x g for 5 min. If the supernatant was very bloody, the lysis buffer wash was repeated. Otherwise the supernatant was taken off and the preadipocyte pellet resuspended in 2mL 10% Dimethyl sulphoxide (DSMO) in a 15% foetal calf serum mix containing penicillin (100units/mL) and streptomycin (100μg/mL) and aliquoted into cryogenic vials. Samples were stored at -80°C overnight and then removed into liquid nitrogen.

2.2. Adipocyte cells culturing methods

After the final centrifugation, step aliquots of 1 mL of adipocyte were resuspended into warm $25cm^3$ flasks each containing 5ml DMEM/F-12 (containing 15% foetal calf serum (FCS) and Dulbecco's minimal essential medium, both from Gibco^{BRL} (Life Technologies™, Paisley, UK) containing 1% transferrin, penicillin (100units/mL) and streptomycin (100μg/mL) (Sigma, Dorset, UK). The final cell concentration is estimated approx 500,000cell/mL as determined by haemocytometer analysis. At this stage, various treatments were added (see individual chapters) and at least two flasks were left untreated as to act as controls to be compared with treated cells.

2.3. Sample preparation for Western blot analysis

2.3.1. Protein Extraction Method (RIPA)

This method was used to extract protein from whole AT or cells for use in Western Blot analysis. For deep frozen adipose tissue or adipocytes, frozen fragments were broken or cut off with a scalpel from the remainder sample under sterile conditions, diluted in 600μL of RIPA buffer (Appendix I) and sequestered. After transfer into eppendorfs and flash freezing in liquid nitrogen, thawed samples were centrifuged at 25,000 g for 20 min at 4°C. Subsequently, the liquid below a fat layer was transferred to clean labelled eppendorfs by using a fine bore needle and syringe. Samples were stored at -80°C till further use. Their concentration was estimated with spectrometry.

Treated adipocytes and preadipocytes were treated in a similar manner. The medium and liquid mix were transferred into 15mL tubes and centrifuged at 140 x g for 30 s. The infranatant (medium) was removed and 500μL of the remainder sample containing adipocytes transferred into eppendorfs with 200μL of RIPA (containing ALLN) (100μL of RIPA for preadipocytes). The samples were flash frozen, thawed, centrifuged at 25,000 x g for 30 min at 4°C and the infranatant transferred to eppendorfs as described above.

2.3.2 Quantification of protein content

The BioRad method is a cuvette reaction using a BioRad DC (detergent compatible) kit containing reagents S (alkaline copper tartrate), A (surfactant solution) and solution B (dilute folin reagent) (BioRad, Hercules, California, USA) (Peterson 1979) (Appendix I). It was used for determination of protein content of extracted samples derived from various adipose tissue depots

(subcutaneous thigh and abdomen and omental fat), treated and un-treated adipocytes and preadipocytes. To ensure a homogenous specimen, all samples were thoroughly thawed and vortexed and 3μL pipetted into a polypropylene cuvette (Starstedt, Germany) and 125μL of a pre-made mixture of 20μL reagent S and 980μL reagent added. Finally, 1mL of solution B was added to each cuvette and the mixture incubated for 15 min at room temperature (RT). At the same time, a standard curve of optical density to protein concentration was constructed using known dilutions of bovine serum albumin (BSA, First Link LTD, UK) (2μg/mL) in the range 0-100μg/mL. Protein concentrations were calculated (μg/3μL). The optical density of protein samples were then analysed on a 6505 UV/VIS spectrophotometer (Jenway, UK) at a wavelength of 650nM.

2.4. Western blot analysis

The general principle of a Western blot is to identify a specific protein of a certain size with the help of electrophoresis, after which the protein is blotted into a PVDF™ membrane. In a second stage, an antibody against the protein of interest was added and the antigen/antibody complex visualised by a chemiluminescent assay with the use of a secondary antibody, as described in full detail below.

2.4.1. Preparation of protein samples for electrophoresis

After protein quantification the samples were thawed and aliquoted into eppendorfs and their volume calculated depending on amount required for loading (e.g. 10 -60μg of protein). Loading buffer was added at a minimum ratio of 1:2 of sample versus loading buffer (Table 2.4.1.) and mQH_2O added to

standardise volumes. After vortex and quick spin the centrifuged samples were heated to 95°C for 5 min before loading into electrophoresis gel. Prepared was also a marker (Amersham, UK), which was diluted 1:5 with the same loading buffer.

Reducing loading buffer for Western analysis:

REAGENT	FINAL CONCENTRATION	QUANTITY
Tris HCl (pH6.8) (Biorad, Hercules,USA)	125mM	625μL
SDS (10%) (Sigma, UK)	4%	1000μL
Glycerol (Sigma, UK)	20%(w/v)	500μL
DTT (Sigma, UK)	6.5 x 10^{-3}%M (w/v)	200μL
Bromophenol Blue	2.5x10^{-3}%M (w/v)	125μL
mQH_2O	100%	250μL

Table 2.4.1.: Contents of reducing loading buffer. Strong reducing loading buffer: contains additional ß-Mercapthoethanol 6μL per sample. Non-reducing loading buffer: without addition of DTT.

2.4.2. Electrophoretic protein separation

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins. In order to optimise for individual proteins and depending on its size, various gel concentrations were used alongside 4.5% stacking gel, pH 6.8, *e.g.*, 8%-15% of resolving gel, pH 8.8. The recipes for each gel are listed in Table 2.4.2. The gel casting unit was assembled according to the manufacturers' instructions and water tightness tested after the glass plates were slotted in. The resolving gel was prepared as outlined above and poured to 2/3 of the total height of the plates. To enhance polymerisation and reduce meniscus

formation about 3mm of milliQ treated water (mQH_2O) was layered over the gel with help of a syringe.

RESOLVING GEL:

REAGENT	CONCENTRATION	QUANTITY FOR 10% 10X10cm GEL	QUANTITY FOR 12% 10X10cm GEL	QUANTITY FOR 15% 10X10cm GEL
1.5M Tris-HCl, 0.4% SDS (pH 8.8)	10% (w/v)	5.2mL	5mL	5mL
mQH_2O	50 %	7.8mL	6.78mL	4.8mL
Ammonium Persulphate	3.3×10^{-2}% (w/v)	100μL	100μL	100μL
TEMED	3.3×10^{-4}% (v/v)	10μL	10μL	10μL

STACKING GEL:

REAGENT	CONCENTRATION	QUANTITY FOR 10X10cm GEL
30% Acrylamide, 0.8%Bis-Acrylamide (37.5:1)	4% (w/v)	1.3mL
0.5M Tris-HCl, 0.4% SDS (pH 6.8)	0.125M	2.5mL
mQH_2O	60%	6.1mL
Ammonium Persulphate	0.1%(w/v)	50μL
TEMED	5×10^{-4}% (v/v)	10μL

Table 2.4.2.: Quantities of reagents that are contained in resolving gel and stacking gel.

The stacking gel was prepared according above recipe and poured over the resolving gel once set. Combs were inserted to created wells (7mm thickness, 15 teeth of 1cm wide each or 10 teeth of 15cm each). Air bubbles were minimized by introducing the comb at an angle and any loss of gel was immediately replaced. On setting, the comb was removed and wells were vigorously flushed with water in a syringe.

Gels encased within the glass plates were transferred to a tank containing 1x electrode buffer (Appendix I), and secured into place, creating two separate reservoirs, allowing ionic movement only through the gels. Wells were flooded and ensured there were no leakages between the two reservoirs. Samples were then loaded using 'duck billed tips' (Fisher, UK). With each set of samples, a marker was loaded (minimum 1 lane per gel) to provide a visual protein size record with which protein samples could be compared. Samples were then resolved by electrophoresis (140-200V) for 1-2 hr depending on the concentration of gel used.

2.4.3. Electrophoretic transfer ('blotting')

Immobilon-P™ membranes (0.45mm) (Millipore, Bedford, Massachusetts, USA) were briefly immersed in 100% methanol (Fisher Scientific, UK), washed in mQH_2O for 1 min and soaked in transfer buffer (Appendix I) until use. Alternatively, for smaller proteins, Westeran-S membrane (0.2mm) (Schleicher&Schuell, Bioscience, Germany), was prepared without water immersion. After completion of electrophoresis, stacking gels were separated from the resolving gel and discarded. The resolving gels were soaked in transfer buffer for 10 min. Fibre pads and filter paper sets (2 sets per gel), which were soaked in transfer buffer for at least 30 min were sandwiched in the following fashion: filter paper was laid over a saturated fibre pad and the resolving layered carefully onto the filter paper; this was topped with the permeabilized membrane, ensuring that no bubbles interrupted the contact between the gel and membrane; if necessary, more transfer fluid was poured over to flush away bubbles. Finally, a layer of saturated filter paper followed with a fibre pad was

added to complete this 'sandwich', which was then inserted into casings and put into a tank containing 2.5L of transfer buffer. Proteins were then transferred with the help of electrophoresis onto the membrane (400mA constant amperage, for 1 hr).

2.4.4. Primary antibody application

Membranes were removed from the stack and the edge trimmed to mark orientation and membrane number. Membranes were incubated with 10% non-fat milk solution (Marvel Milk Powder, Premier Brands, Merseyside, UK) diluted in 0.5% PBS-polyoxyethylene sorbitan monolaurate (Tween 20 (0.1% (v/v), Sigma, Dorset, UK) on an orbital shaker for at least 1 hr or overnight in the cold room at 4°C to block non-essential proteins. Membranes were then rinsed repeatedly in PBS and finally in 1% PBS-Tween20 (PBS-T). Primary antibody was prepared in sufficient quantity to cover the surface of the membrane at a concentration of 0.05% PBS-T and incubated with the membrane in a 50mL falcon tube at 4°C overnight. After approximately 14 hr at 4°C, membranes were washed three times in 10mL PBS (120mM, pH 7.6) and then finally in an excess of 1% PBS-T (120mM). Filters were then returned to the shaker for three 10 min washes in excess 1 % PBS-T (120mM). Alternatively, if the membranes were blocked overnight, the primary antibody incubation period was shortened to about 1 hr at RT and then washed accordingly.

2.4.5. Secondary antibody application

The secondary antibody was made up in 0.5% PBS/PBS-T and membranes incubated at RT for 1 hr on an orbital shaker at medium speed (80-

100cycles/min). The membranes were rinsed three times in PBS (120mM, pH 7.6) and after each of the three subsequent 10 min washes in excess 1% PBS-T.

2.4.6. Immunodetection of antibody labelled proteins

The function of the secondary antibody is to make specific proteins visible by acting as a catalyst for luminol, a substrate that transmits light, which is provided by an ECL Western blotting detection system and its stronger version like ECL plus and ECL advance (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The secondary antibody binds to the primary antibody that is attached to the protein under scrutiny. Chemiluminescence was achieved by a reaction, which is based on the oxidation of the cyclic diacylhydrazide luminol (see Figure 2.4.6.). The addition of HRP and peroxide catalyses the oxidation of the lumigen PS-3 acridan substrate generating acridinium ester intermediates, which produce a sustained, high intensity chemiluminescence with reaction with peroxide under slightly alkaline conditions The light emitted was then registered by application of photographic X-ray film.

The Membranes were removed from the secondary antibody and washed in an excess of PBS (120mM, pH 7.6) followed by three 10min washes with 1% PBS-T (120mM). They were placed protein side-up on saran wrap. Equal volumes of ECL solution A (tris buffer) and B (acridin solution in dioxane and ethanol) (or appropriate mixtures of ECL-plus, or ECL advance solutions to allow 3mL per 4cm^2 area of membrane) were mixed and each membrane covered. This was incubated for 1 min (or 5 min for ECL-plus and for ECL advance), with excess

ECL poured away and the corner of the membrane blotted on tissue paper to remove excess ECL solutions.

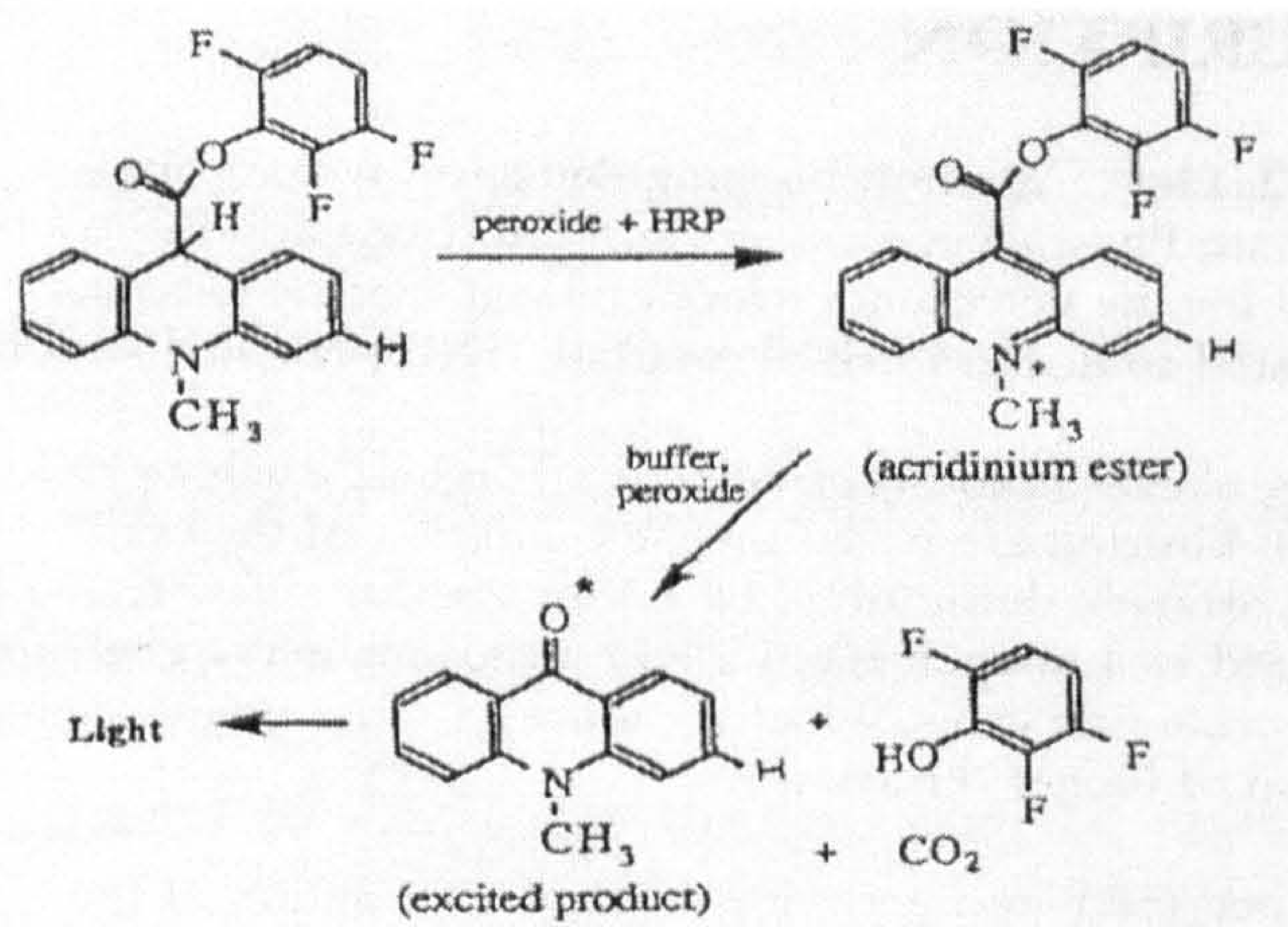


Figure 2.4.6.: Chemiluminescent reaction of Lumigen PS-3 with horseradish peroxidase (Adapted from Isacsson, 1974).

Membranes were placed between two sheets of clear plastic, further excess ECL removed with tissue paper and placed into a film cassette. The filters were then exposed to photographic X–ray film (Kodak, UK) for varying times (15 s – 12 hr).

2.4.7. Quantification of Western blot bands

The density of the bands on the autoradiographs were quantified by gel blot analysis using UVP Gel Blot Analysis System (UVP, UK) and statistically analysed, as detailed in the individual results chapters.

2.5. RNA Isolation and preparation for PCR

2.5.1. Extraction and quantification of RNA.

In order to extract mRNA from deep frozen adipose tissue, a piece of 5g was taken, placed into 1mL of QIAzol Lysis Reagent and disrupted with a homogeniser. In preparation for RNA extraction of adipocytes, a freshly isolated and centrifuged sample (see above) of 200µL was taken from the densely packed adipocyte layer and placed into sterile 1.5mL eppendorf tubes with 800µl RNALater™ (Ambion, AMS Biotech, UK). Adipocytes were then kept at 4°C overnight and transferred to -70°C for storage. For the extraction, the samples were carefully thawed and the top layer containing adipocytes taken off the RNALater™ fluid and similarly placed into 1mL QIAzol Lysis Reagent and disrupted with a homogeniser.

2.5.2. RNA isolation and purification.

Total RNA was isolated from whole AT using a column-based method, according to the manufacturer's instructions (RNeasy Lipid Tissue Mini Kit, Qiagen, UK). After RNA elution, the sample was digested with 7µL (1000units/mL) of DNase enzyme (DNase Kit, Sigma, UK) and 7µL reaction buffer (DNase Kit, Sigma, UK) to remove contaminating genomic DNA. These reactants were incubated for 15 min at standard RT. The reaction was stopped through the addition of 7µL stop solution (50mM EDTA) (DNase Kit, Sigma, UK) and subsequently samples were incubated at 70°C for 10 min. (Solutions are outlined in appendix I).

2.5.3. RNA quantification

The total RNA concentration of each individual sample was quantified using a spectrophotometer (wavelength 260nm, Nanodrop, Labtech, UK) and the RNA assessed using the absorbance ratio 260/280nm. Protein contamination of RNA was calculated by measuring optical density of each sample at 260nm (OD_{260}) (wavelength for RNA detection) and 280nm (wavelength for protein detection). The ratio of these two readings is an indication of RNA concentration. The conversion factor for RNA is 0.040μg/μL per OD_{260} unit and the quantity of undiluted RNA was 1/μL, with:

$$\text{Concentration } (\mu g/\mu L) = A_{260} \times \text{dilution factor} \times \text{conversion factor}$$

2.5.4 Reverse transcription of mRNA.

MRNA (1μg) from each sample was pipetted into a sterile microcentrifuge tube along with 0.5μL of random hexamers mixed in equal quantities. To standardise, nuclease free water was then added to each aliquot to make up a final volume of 11μL and samples heated to 70°C for 10 min. A reverse transcriptase (RTn) master mix was pre-mixed (see Table), containing appropriate quantities of RNase inhibitor enzyme (RNasin), avian myeloblastosis virus reverse transcriptase enzyme (AMV), reaction buffer (10X), magnesium (Mg^{2++}, 25mM stock), deoxynucleoside triphosphates (dNTPs containing guanine, thymine, adenine and cytosine) (Promega, UK), and nuclease free water sufficient for the chosen number of RTn reactions. After 10 minutes of incubation, 10μL of RTn master mix was added to each sample of RNA. After a vortex and centrifugation samples were heated to 37°C for 1 hr, after which the reaction was quenched by

a consecutive incubation at 95°C for 5 min to denature the enzymes. The resultant cDNA product was stored at -20°C for further use.

Contents of Reverse Transcription Master Mix	Concentration in final 20μL Volume	Quantity added (μL)
Reverse transcription 10x Reaction buffer (Mg^{2++} free)	1x (v/v)	2.0
Mg^{2++} (25mM)	5mM	4.0
dNTPS (10mM)	1mM	2.0
AMV (20u/μL)	0.5u/μL (v/v)	0.5
RNasin (40u/μL)	1u/μL (v/v)	0.5
Nuclease free H_20	5%	1.0

Table 2.5.4: Quantities if substances for the reverse transcription master mix. The final volume of cDNA sample is 20μL where 1μL equals 50ng of single stranded cDNA.

2.5.5. Principles of the polymerase chain reaction

The theory of cDNA synthesis from DNA is discussed in Appendix II. Genes were amplified using the polymerase chain reaction (PCR) which is based on cycling temperatures, which allows the amplification of millions of copies of the gene of interest.

2.5.6. Quantitative Real-Time PCR

The real-time PCR reaction was performed with volumes of 25μL on 96 well plates, in a reaction buffer containing Taqman universal PCR master mix, 3mM $Mn(Oac)^2$, 200pM dNTPs, 1.25units ampliTaq gold polymerase, 1.25units ampErase UNG, 100-200nmoL Taqman probe, 900nmoL primers and 25-125ng.

Thermal Cycler	Times and Temperatures			
	First Steps		1 of 44 cycles	
			Melt	Anneal/Extend
ABI Prism 7500 Sequence Detector	Hold	Hold	Cycle	
	2 min 50°C	10 min 95°C	15 s 95°C	1 min 62°C

Table 2.5.6.: Rt-PCR reaction conditions as pre-set on the ABI 7500.

All reactions were multiplexed with the housekeeping gene for the 18S ribosomal subunit, provided as a pre-optimised control probe (PE Biosystems, UK) enabling data to be expressed in relation to an internal reference which allowed for differences in RT efficiency. Data were obtained as the cycle number at which logarithmic PCR plots cross a calculated threshold line or the Ct value. In accordance with the manufacturer's guidelines ΔCt values were determined (ΔCt = Ct of the target gene minus Ct of the housekeeping gene). Measurements were carried out in triplicate and the target gene probes were labelled with the fluorescent label FAM, and the housekeeping gene with the fluorescent label VIC. The settings of the thermal cycler are described in table 2.5.6. and the required solutions for quantitative real time PCR are given in appendix I.

2.5.7. Data handling and statistical analysis for RNA

To exclude a potential bias due to averaging data, which had been transformed through the equation $2^{-\Delta\Delta Ct}$, all statistics were performed at the ΔCt stage. For both mRNA and protein findings, statistical analysis was undertaken using an unpaired Student's t-test. The threshold for significance was $p<0.05$. Data in the text and figures are presented as mean ± SEM.

2.6. Microarray analysis

2.6.1 Outline

After RNA extraction and quantification as described in section 2.5.1. RNA was reverse transcribed using a T7-Oligo(dT) Promoter Primer in the first-strand cDNA synthesis reaction and second- strand cDNA synthesized. The double-stranded cDNA was then purified and used as a template for the *in vitro* transcription (IVT) reaction which was carried out in combination of T7 RNA Polymerase and a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labelling. The biotinylated cRNA targets were then cleaned up, fragmented, and hybridised to GeneChip expression arrays.

2.6.2. Preparation of controls

To provide exogenous positive controls and to enable monitoring of the entire eukaryotic target labelling process, a set of poly-A RNA controls is supplied in the GeneChip Eukaryotic Poly-A RNA Control Kit. Each of this probes contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys, phe, thr,* and *dap*). These poly-A RNA controls and the polyadenylated transcripts for the *B. subtilis* genes are pre-mixed at staggered dilutions before being amplified, labelled together with the samples and spiked directly into RNA samples to achieve the final dilutions. Analysis of the hybridisation intensities of these controls on GeneChip arrays help to monitor the labelling process independently.

2.6.3. First-strand cDNA synthesis

Extracted RNA (5µg) was mixed with diluted poly-A RNA controls and T7-Oligo(dT) Primer and water to a final volume of 12µL in 200µL centrifuge tubes. After being briefly centrifuged and incubated for 10 min at 70°C, the samples were cooled at 4°C for at least 2 min and briefly spun.

RNA/T7-Oligo(dT) Primer Mix Preparation		**Addition of :**		
Extracted sample RNA	variable	**First strand master mix**		**Total volume**
Diluted poly-A RNA controls	2µL	5X Buffer	4µL	
T7-Oligo(dT) Primer	2µL	DTT (0.1M)	2µL	
RNase-free Water	variable	dNTP (10mM)	1µL	
Volumes of components	**12µL**		**7µL**	**19µL**
Addition of:			**SuperScript 2**	**1µL**
Total volume				**20µL**

Table 2.6.3.: Reagents for first-strand cDNA synthesis and their volumes

A First-Strand Master Mix was prepared separately (Table 2.6.3.) of which 7µL was added to each RNA/T7-Oligo(dT) Primer mix for a final volume of 19µL. The tube was flicked a few times and centrifuged briefly, and then immediately incubated at 42°C for 2 min. 1µL of SuperScript II was then added to each sample for a final volume of 20µL The tubes were mixed and centrifuged briefly before immediately being incubated at 42°C for 1 hr. After incubation the tubes

were cooled to 4°C and briefly spun before immediately continuing with second strand cDNA synthesis.

2.6.4. Second-strand cDNA synthesis

Second-strand master mix (130µL), which was prepared as described below, was added to the above sample, mixed, briefly centrifuged and incubated at 16°C for 2 hr. T4 DNA polymerase (2µL) was added to each tube and incubated for a further 5 min at 16°C. After incubation, the reaction was stopped with addition of 10µL EDTA (0.5M).

Above sample		**Addition of:**			
		Second strand Master Mix			
		5X Buffer	30µL	**T4 DNA polymerase**	
		E. coli DNA ligase	1µL		**EDTA (0.5M)**
		E. coli DNA Polymerase I	4µL		
		RNase-free Water	91µL		
		dNTP (10mM)	3µL		
		RNase H	1µL		
Volumes of components	**20µL**		**130µL**	**2µL**	**10µL**
Total volume					**162µL**

Table 2.6.4.: Reagents of second-strand cDNA synthesis and their volumes

2.6.5. Cleanup of double stranded cDNA

To each of the above, cDNA preparation 600µL of cDNA binding buffer was added and briefly vortexed. This mix was loaded onto a cDNA cleanup spin

column and centrifuged for 1 min at 10,000 x g, the flow through was discarded and the column transferred to a new collection tube. After this, 750µL of cDNA wash buffer was added, the column was centrifuged for 1 min at 10,000 x g and the flow through discarded. The collection tubes were then centrifuged again for 5 min at 10,000 x g with their caps opened to allow drying of the membranes. The spin columns were then transferred to fresh 1.5mL collection tubes and 14µL of cDNA elution buffer added directly to the membranes. The tubes were incubated for 1 min at RT before spinning again for 1 min at 10,000 x g to elute and the collection tubes with the sample stored.

2.6.6. Synthesis of biotin-labelled cRNA

To 12µL of each sample from above section, 28µL a biotin labelling master mix, which was prepared according to table 2.6.6., was added, mixed and briefly spun before being incubated for 16 hr at 37°C.

Above sample		**Addition of:**	
		Biotin labelling master mix	
		10X IVT Labelling Buffer	4µL
		IVT Labelling NTP Mix	12µL
		IVT Labelling Enzyme Mix	4µL
		RNase-free Water	8µL
Volumes of components	**12µL**		**28µL**
Total volume			**40µL**

Table 2.6.6.: Biotin-labelled cRNA master mix preparation

2.6.7. Cleanup of biotin labelled cRNA

RNase free water (60µL) was added to each sample and briefly vortexed after which 350µL of IVT cRNA binding buffer was added and mixed. 250µL of 100% ethanol was added, the tubes were mixed well again. This mix consisting of a volume of 700µL was added to the IVT cRNA cleanup spin column and centrifuged for 15 s at 10,000 x g, the flow through was discarded. The column was then transferred to a fresh collection tube and 500µL IVT cRNA wash buffer was added before spinning again for 15 s at 10,000 x g and discarding the flow through. Next 500µL of ethanol (80%) each was added and the collection tubes centrifuged at 10,000 x g for 15 s and the flow through discarded. The tubes were then spun again for 5 min at 10,000 x g with their caps open to allow the drying of the membranes. The columns were then transferred to fresh 1.5mL collection tubes and 11µL of RNase free water added directly to the column membrane, the columns were then spun at 10,000 x g for 1 min and repeated with a second volume of 10µL of RNase free water to be added to the column membrane and the eluted fluid collected into the same collection tube.

2.6.8. Fragmenting the cRNA for Target Preparation

The cRNA was finally quantified using the nanodrop ND-1000 spectrophotometer as described previously. For the fragmentation reaction 20µg of each cRNA sample was mixed with 8µL of 5X buffer to which RNase free water was added to achieve a final volume of 40µL then incubated for 35 min at 94°C and placed on ice.

2.6.9. Target Hybridisation

Fragmented and labelled cRNA (15μg) for each sample was used for the hybridisation cocktails (Table 2.6.9.). The 20X GeneChip eukaryotic hybridisation controls were incubated for 5 min at 65°C before addition to ensure complete resuspension of cRNA. After its mixture the samples were incubated for 5 min at 99°C.

Hybridisation cocktail	
Fragmented and labelled cRNA (15μg)	variable
Control oligonucleotide B2 (3nM)	5μL
20x Eukaryotic Hybridisation	15μL
Controls(*bioB, bioC ,bioD ,cre*)	15μL
2X Hybridisation mix	150μL
DMSO	30μL
Nuclease free water	variable
Total Volume	**300μL**

Table 2.6.9.: Reagents for hybridisation cocktail

Meanwhile the arrays (Affymetrix Human Genome U133 Plus 2.0) were prepared for which they were wet with 200μL of pre-hybridisation mix by filling through one of the septa. The arrays were incubated for 10min at 45°C with rotation in the hybridisation oven. The hybridisation cocktail was also transferred to a 45°C incubation for 5 min before being centrifuged at 10,000 x g for 5 min in order to collect any insoluble material. The arrays were then removed from the hybridisation oven, vented with a clean pipette tip and the pre-hybridisation mix was removed. The arrays were refilled with 200μL of the clarified hybridisation cocktail and were placed back into the hybridisation oven set to 45°C and rotated at a rate of 100 g for 16 hr.

2.6.10. Array completion

Following hybridisation, the arrays were removed from the oven, vented and the hybridisation cocktail removed and replaced by 250µL of wash buffer A. For each array one 1.5mL amber microcentrifuge tube containing 600µL of stain cocktail 1 was placed into the fluidics station; in addition clear 1.5mL microcentrifuge tubes containing 600µL of stain cocktail 2 and 800µL array holding buffer were added for each array. The probe arrays were inserted into the designated module of the fluidics station and the fluidics protocol FS450_0001 run. On completion of the wash and stain protocol, the arrays were removed from the fluidics station and sequentially scanned using the Affymetrix GeneChip scanner 3000.

2.7. Immunohistochemistry

Immunohistochemistry was used for assessment of the presence of specific proteins in various tissues as outlined below.

2.7.1. Embedding tissue in wax

Sections of tissue were processed in agreement with local ethical committee guidelines. Cubes of ~1.5cm^3 tissue (e.g. adipose tissue) were fixed by placing them in an excess of 10% formalin (Sigma, UK) diluted in mQH_2O for between 7 and 10 days (depending on size of block). Very small, thin blocks of tissue were fixed within 24-48 hr. Tissue was then dehydrated in varied concentrations and changes of ethanol for the following times;

70% Ethanol 2-3hr x 2 changes

90% Ethanol 2-3hr x 2 changes

100% Ethanol 4-8hr x 2-3 changes

The tissue was cleared of ethanol using 100% xylene (Fisher, UK) for between 1-5 hr (or until transparency was achieved) and xylene changed twice within the incubation time. The subsequent 8hr impregnation period included three separate dippings of tissue into molten wax (2-30°C above the melting point of wax) after which the tissue was cut into 3 micron thin slices and mounted onto electrostatically charged slides (Superfrost plus Slides, BDH Laboratory Supplies, Poole, UK).

2.7.2. De-waxing of tissue sections

Labelled slides were de-waxed by complete immersion in 100% xylene (Fisher, UK) with two 5 min washes; this was followed by two 3 min washes in IMS alcohol (100%). All slides were then re-hydrated by washing thoroughly under running tap water. Individual slides were placed in a coplin jar and treated with methanol-hydrogen peroxide (0.6%) to block endogenous peroxidase activity. Slides were then rinsed repeatedly in excess water. Slides were assembled in the Sequenza define (Shandon, UK) and rinsed in excess PBS solution (0.05mol/L, pH 7.6). Sections were blocked using 10% donkey serum (100μL) (DS) (ABC detection Kit, The Binding Site, UK) diluted in PBS for 30 min at RT. Slides were rinsed with PBS solution (0.05mol/L, pH 7.6).

2.7.3. Incubation with primary and secondary antibody

The labelled slides were incubated with 100μL of the primary antibody solution for 1hr at room temperature. The solution contained 10% normal swine serum in PBS solution (0.05mol/L, pH 7.6) In order to produce a negative control, the primary antibody was omitted for the control slides. All tissue sections were rinsed with excess of PBS solution, the same which was used to dilute the secondary 'universal' antibody to 1:100 in PBS solution (0.05mol/L, pH 7.6). The latter was added to the slide sections for 30 min. The slides were rinsed again with PBS solution (0.05mol/L, pH 7.6) and incubated with avidin-biotin complex (ABC reagent) for 30 min. The ABC reagent was prepared 30 min before use (containing 1mL PBS (0.05mol/L, pH 7.6), 10μL avidin (Reagent A) and 10μL biotin (Reagent B), which is used to amplify and detect the secondary antibody. Following incubation the slides were placed on a staining tray and immersed into the DAB/urea mix (containing 10mg 3,3'-diaminobenzidine (DAB) and urea (0.17mg/mL) (Sigma, UK) dissolved in 20mL PBS) and incubated for 5-6 min or until a brown stain appeared. The reaction was stopped by rinsing with tap water.

2.7.4. Counter-staining of sections and dehydration

Sections were counter-stained with excess Mayer's haematoxylin (BDH, Poole, UK) for 30 s. The reaction was terminated by fully immersing slides in tap water. Slides were immersed for 5 min in 2 sequential tanks of IMS alcohol (100%), followed by 2 min submersion in two changes of xylene (100%). Slides were mounted in a xylene-based mountant (DPX; BDH, Poole Dorset, UK) by

placing a drop onto a glass coverslip and covering section. Pressure was applied to expel air bubbles from the coverslip and allow DPX to fully cover the section.

2.7.5. Peptide Blocking

Pre-absorption with immunising peptides was used to demonstrate the specificity of the antibodies. This was done by pre-incubation of the primary antibody for 1 hr with a peptide containing the epitope which was originally used to raise the antibody. This was performed in a solution containing 10% normal swine serum in PBS solution (0.05mol/L, pH 7.6) at room temperature. After 1 hr the antibody/peptide mixture was applied to sections in the sequenza slide rack and incubated as described above.

2.7.6. Image acquisition

The slides with stained mounted sections were analyzed under a standard light microscope; for magnification above x1000, an oil immersion technique was used. A real time digital image capture system (Amersham Biosciences, UK) was used to photograph sections.

2.8. Separation of serum and plasma from blood

In preparation of serum samples, whole blood was drawn directly into a BD-vacutainer® (BD) serum tube containing silica clot activator. Each sample was left at room temperature for 30 min to allow the blood to clot. Once clotted, the samples were centrifuged at 700 g for 15 min at room temperature. Supernatant was carefully drawn off and stored in labelled 1.5mL tubes at -70°C. A similar procedure was followed for plasma (vacutainer ® (BD) plasma tube.

2.9. Enzyme linked immuno-absorbant assay (ELISA)

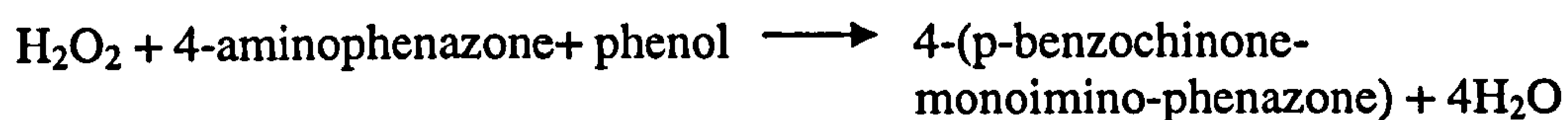
The following describes the basic procedure of ELISA, which varied according to antibody utilized. The microtiter ELISA plate is coated with a monoclonal human antibody, which is captured by corresponding protein molecules of the samples. The samples are loaded in appropriate dilutions according manufacturers recommendation. In the case of cerebrospinal fluid analysis, the required dilution ratio was judged by precedent assessment of different dilutional states and their agreement with the standard curve. After loading of standards, quality control and samples a further antibody was added and the plate was incubated for 2 hr. The immobilized antibody was treated with an enzyme solution, the free enzyme solution was removed after a further incubation period of usually 30 min and the activity of remaining and immobilized enzyme solution bound to the antibodies sample complex measured with help of chemo-luminescence. These were derived from enzyme activity after activation through a light sensitive substrate. A spectrophotometer was used to measure the intensity of absorbance of the samples. The values are then interpolated into a standard curve derived from samples with known protein concentrations generated in the same assay and concentrations of the protein in unknown samples obtained. Further details are given in the individual chapters.

2.10. Glucose estimation

Glucose was collected in vacutainers containing Fluoride and EDTA and immediately centrifuged at 3500 x g for 20 min and consequently assessed in the lab with an enzymatic colourimetric assay, the Glucose GOD Method. Glucose was oxidised by glucose-oxidase (GOD) to gluconolactone and hydrogen

peroxide. H_2O_2 was oxidised with phenol and peroxidase (POD) 4-amino-phenazone to 4-(p-benzochinone-monoimino)-phenazone, which is a red dye and its colour intensity and the photometrical signal directly proportional to the glucose concentration.

Glucose + O_2 + H_2O $\longrightarrow$ gluconolactone +H_2O_2



H_2O_2 + 4-aminophenazone+ phenol $\longrightarrow$ 4-(p-benzochinone-monoimino-phenazone) + $4H_2O$

Chapter 3

Adiponectin and resistin in human cerebrospinal fluid and adiponectin receptors in the human hypothalamus

3.1. Introduction

Previous studies of the adipokine leptin have highlighted its role as a mediator in the crosstalk between adipose tissue and the brain in regulating energy homeostasis. Leptin's anorexic action is mediated primarily through neuronal targets in the arcuate nucleus within the hypothalamus where it inhibits NPY neurons. Recent studies also highlight that adiponectin appears to be involved in altering energy expenditure and thermogenesis (Qi, 2004) and resistin could affect hypothalamic feeding circuits (Brunetti, 2004). mRNA expression of resistin has also been found in the arcuate nucleus of mice (Morash, 2002). Adiponectin is a multimeric and multi functional protein, the levels of which are reduced in states of obesity and type 2 diabetes mellitus (T2DM); its effects appear mediated by adiponectin receptors (Wolfe, 2004; Berg, 2005; Yamauchi, 2003). In contrast, some reports are showing increased resistin in obesity and T2DM (McTernan, 2006), whilst its receptors remain unknown. To date, however, little is known about the potential central action of either of these adipokines in humans. Many studies have investigated leptin, its pleiotropic functions - including its role in central energy homeostasis - and its passage through the human BBB. It can activate numerous signalling pathways (Fruhbeck, 2006) and, in particular, mediates its anorexigenic action through the AMPK pathway in the liver as well as the hypothalamus (Minokoshi, 2004). Furthermore, it has been shown that leptin can stimulate the sympathetic system centrally to produce feedback of fat mass by induction of lipolysis (Rayner, 2001). Leptin is a hormone which is predominantly secreted by adipocytes and also a key messenger to central energy homeostasis so it is likely that other adipokines contribute to the crosstalk between AT and the brain.

The aim of this study was to examine the potential central role of resistin and adiponectin in humans. Matched paired serum and human CSF samples from male and female patients were compared in order to explore the relationship between CSF and the corresponding serum levels and to determine the CSF/serum ratio as a measure of efficient uptake into the CSF. This study also considered possible correlations between adiponectin, resistin and leptin in CSF with age, gender, BMI and HOMA index. Finally, this study determined the expression and distribution of adiponectin receptors in the human hypothalamus. These issues have become particularly important in the light of recent report that adiponectin is not present in human CSF (Spranger, 2006) and exogenous adiponectin does not enter the BBB by endocytosis of rodent RBE4 cerebral microvessel endothelial cells (Pan, 2006).

3.2 Materials and Methods

3.2.1 Subjects

Serum and CSF matched samples were obtained from adult age and BMI matched subjects (men: Age: 69.8(mean±SD)8.6years, BMI: 29.4±3.4kg/m^2, n=20; women: Age: 69.4±4.3years, BMI: 27.3±4.8kg/m^2, n=19) undergoing elective surgery, under spinal anaesthesia and fasted for at least 8 hrs (Table 3.3.4.). All samples were collected with the approval of the local ethics committees and with the informed written consent of all study subjects who were personally consented. They were approached after being identified as candidates for spinal anaesthesia and agreement of their individually assigned anaesthetist and surgeon. Exclusion criteria were malignancy, acute or chronic renal or liver disease, neurological disorders, the use of immunosuppressants, current or recent use of systemic high dose corticosteroids, antibiotics or weight modifying medication. Patients with C-reactive protein (CRP) levels above 10 mg/dL were also excluded. Surgical interventions consisted mainly of orthopaedic procedures: 10 knee and 12 hip replacements, 4 arthroscopies, and the remainder 16 were minor gynaecological and urological procedures. Patient characteristics are shown in Table 3.3.4..

A fasting blood sample (5mL) was taken at the time of venopuncture and a clear CSF sample (2mL) collected before the spinal anaesthetic agent was injected. Six patients had type 2 diabetes (two on daily insulin treatment) and three had impaired glucose tolerance (IGT). The T2DM subjects were on glucose/insulin infusion at the time of cannulation and could not be considered for HOMA-IR calculation. The HOMA index was used as a measure of insulin resistance (see below). Serum and plasma EDTA samples were immediately centrifuged, snap frozen in liquid nitrogen and stored at -80°C. CSF samples were passed through a

0.2micron syringe filter (Schleicher & Schuell, UK), flash frozen and stored at -80°C.

3.2.2. Analysis of serum samples

The serum samples were analysed for the determination of adiponectin (1:500 dilution; Linco Research Inc., Missouri, USA) with the assay limit of 0.78ng/mL, intra-assay %CV was 7.4% and inter-assay variability was 2.4-8.4%. Serum resistin levels were analyzed by ELISA (1:5 dilution; Quantikine, R&DSystems, UK). Assay limits were noted between 0.01-0.055 ng/mL with intra-assay % CV noted as between 3.8-5.3% and inter-assay variability being 7.8-9.2%. The recovery of resistin in serum and cross-reactivity with resistin-like molecules (RELMs) was assessed as previously described (McTernan, 2002).

The leptin ELISA (1:100 dilution; Quantikine, R&D systems, UK) has a minimum detectable value of 7.8pg/mL with an intra-assay precision of 3-3.3%CV and inter-assay variability of 3.5-5.4%CV. The high sensitive CRP ELISA (Life Diagnostics Inc., USA) has a functional sensitivity of 0.1mg/dl, intra-assay precision of 2-7.5%CV and inter-assay variability of 2.5-4.1%CV. An insulin ELISA (Linco Research Inc. Missouri, USA) was utilised with the sensitivity of 2μU/mL; intra-assay %CV was 5.96 and inter-assay variability was 10.3%CV in accordance with the manufacturer's protocol. Glucose levels were analysed using the YSI-2300 STAT PLUS, according to the manufacturer's instructions.

The HOMA2-IR index was calculated using the HOMA calculator (available at: www.dtu.ox.ac.uk/index.html?maindoc=/homa/, University of Oxford, UK). which is suitable for use of insulin values derived by modern ELISA assays.

3.2.3. Analysis of CSF levels

CSF samples were used undiluted for assessment of adiponectin (20μL), resistin (100μL) and (10μL) leptin. The serial dilution of CSF detected adiponectin levels up to a 1 in 4 dilution (86% of value expected 1 in 2 dilution and 80% of value expected 1 in 4 dilution). A similar serial dilution was documented for resistin, serial dilution ranging from undiluted to 1 in 8. The use of neat CSF sample produced results with a recovery of 100%, and therefore deemed most acceptable in this system.

The potential sources of cross-reactivity in the adiponectin ELISA was assessed for C1q (Abcam, Cambridge, UK), collagen type III (Sigma, Poole, UK) and collagen type IV (Sigma, Poole, UK). For this human recombinant (rh) C1q, rh collagen type III and rh type IV were analysed at two concentrations (10ng/mL, 100ng/mL) in either the presence of phosphate buffered saline pH 7.6 (Sigma, Poole, UK) or in combination with CSF. No detectable cross-reactivity occurred between rh C1q, rh collagen type III and rh type IV alone or in combination with CSF samples and adiponectin in this ELISA. The inter- and intra- assay coefficients were evaluated for samples ranging from 12.1-15.9ng/mL CSF adiponectin. The within percentage CV was 2.5% and the between CVs were 6.7, 2.4 and 5.3%. The means between variations were calculated as described in the manufacturer's instructions.

Spiking and recovery of human adiponectin in CSF was further examined. Human rh adiponectin (20ng/mL, 50ng/mL) was added to known concentrations of adiponectin in pooled CSF samples (12.3ng/mL, 15.9ng/mL respectively) and adiponectin content was determined in three separate assays. The percentage of recovery was determined according to manufacturers' instructions. We observed

that recovery ranged from 93 to 97% in CSF, which was within a comparable range as noted for serum according to the manufacturer's detailed assessments.

Spiking and recovery of human resistin in CSF was also addressed. For these studies two concentrations of rh resistin (0.5ng/mL and 2ng/mL) were added to known concentrations of resistin in pooled CSF samples (0.03ng/mL, 0.08ng/mL respectively) and resistin concentration was determined in three separate ELISA assays. The percentage of resistin recovery ranged from 81 to 99%, with a mean of 88% in CSF, again similar recovery for serum findings was noted by the manufacturer as well as from our previous publications (McTernan, 2003).

Blood contamination was excluded with use of the micropore filter described above. The cell count in CSF samples with the haemocytometer (Appendix II) did not show any nucleus bearing cells. Further screening of the CSF samples with the Haemoccult test (Hema-screen™; Immunostics Inc, New Jersey, USA) (Appendix II) test was negative yet the test remained positive with spiking of full blood at a concentration of 1:10,000.

3.2.4. Immunohistochemical staining for adiponectin receptors

Human adipose tissue was obtained from Medical Solutions (Peterborough, UK). Hypothalamic post-autopsy sections were obtained with the approval of the Varna Medical Research Ethics Committee from Varna University, Bulgaria. Paraffin embedded tissue sections were prepared for immunohistochemical staining as described in Chapter 2. Tissue was incubated with primary polyclonal AdipoR1 and AdipR2 antibody (Phoenix Pharmaceuticals, USA) in a dilution of 1:2500 and 1:200 respectively and dual stained with NPY in a dilution of 1:400. Sections

were developed using peroxidase substrate kit VIP® (Vector Laboratories Ltd., Peterborough, UK) for AdipoR 1 and 2 and with diaminobenzidine (BioGenex, California, USA) for NPY. To demonstrate specific binding, the primary antibody was omitted for negative control for each primary antibody individually.

3.2.5. Statistical analysis of results.

Statistics were performed using SPSS version 14 (SPSS UK, Surrey, UK). P values of less than 0.05 were considered as statistically significant. Analysis of normally distributed variables was by linear regression analysis and two tailed Student's t-test. For non-parametric distributions, *e.g.*, HOMA, we used the Spearman correlation. Values are expressed as mean ± standard deviation of the mean, unless otherwise stated.

3.3 Results

3.3.1. Presence of adiponectin and resistin in CSF

The presence of leptin was confirmed in human CSF, but the presence of both adiponectin and resistin were also identified. The figure below shows the relationship with corresponding serum samples. In relation to its serum levels, the CSF adiponectin proportion is much lower (1:1000) than resistin (1:100), however the absolute concentration of adiponectin in CSF is higher than CSF resistin, and in the range of serum resistin. The levels will be discussed further under the individual subheadings.

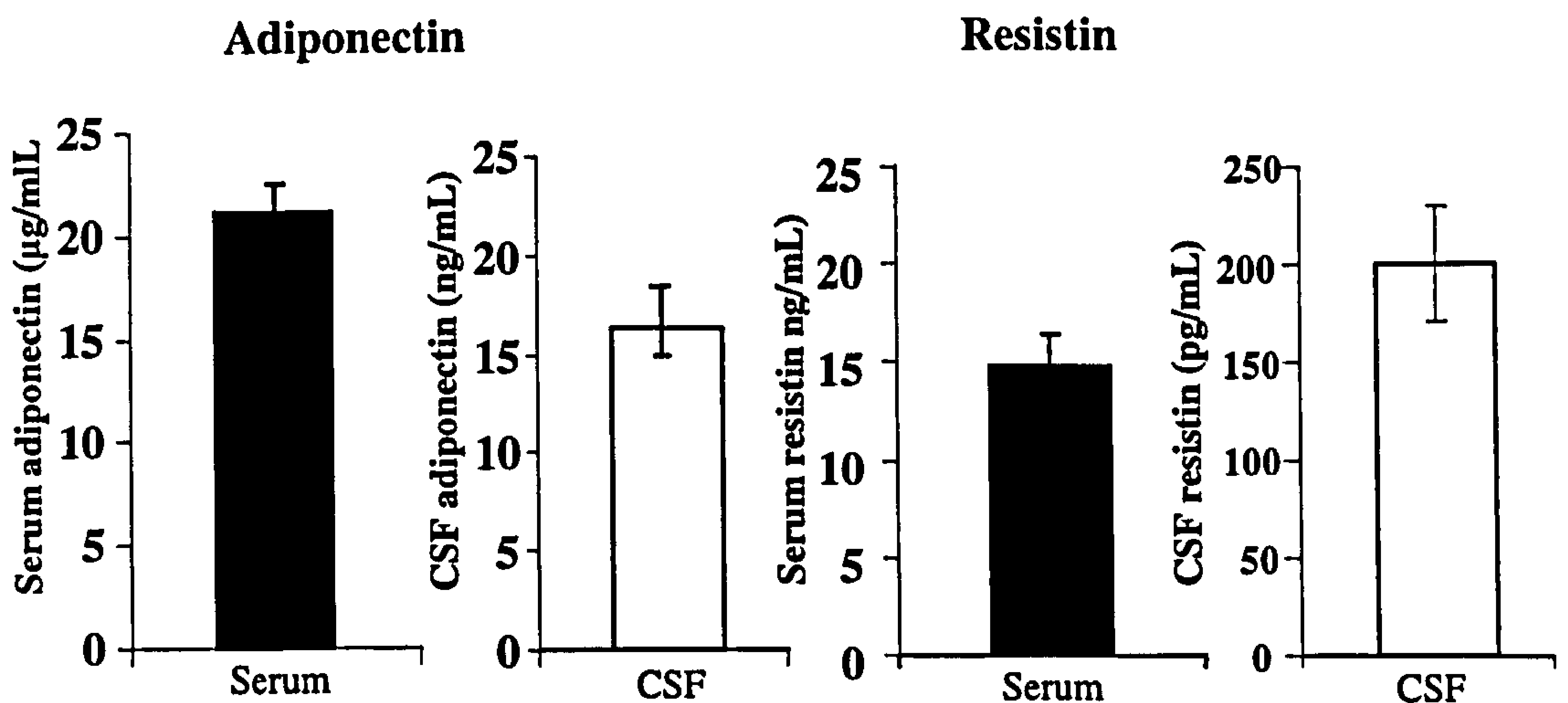


Figure 3.3.1.: Serum adiponectin levels (µg/mL) are 1000 times lower in CSF (ng/mL) and serum resistin levels (ng/mL) are 100 times lower in CSF (pg/mL).

3.3.2. Leptin

3.3.2.1. Serum leptin

Mean serum levels of leptin in this study population were 17.4±2.5ng/mL with significantly higher levels identified in women (22.5±3.7ng/mL versus men: 13±3ng/mL, $p=0.03$). Serum leptin levels were positively correlated with BMI (overall $r=0.52$, $p<0.001$; men: $r=0.75$, $p=0.001$; women: $r=0.82$; $p<0.001$) and

showed no correlation with age. Serum leptin was higher in T2DM than in non-diabetic subjects (28.9±5.8ng/mL versus 12.5±8.7ng/mL, p=0.02).

3.3.2.2. CSF levels of leptin

The CSF levels showed a tendency towards higher leptin levels in women than men but this was not significant (women: 79±9pg/mL versus men: 56±9pg/mL, p=NS). CSF leptin was positively correlated with BMI in men and women (r=0.42, p=0.028) but not with age. In comparison to the observed association of serum leptin with diabetes there was no significant difference of CSF leptin in the T2DM subjects (T2DM subjects: 105±2pg/mL versus non-diabetic subjects: 62±7pg/mL, p=NS).

3.3.2.3. CSF/serum ratio of leptin

To establish the proportion of serum levels which may enter the CSF, we calculated the CSF/serum ratio. There was a negative correlation of the CSF/serum ratio with serum levels, which was similar to previous reports best described by a logarithmic association (r=0.62, p=0.001) (Figure 3.3.5.1.A). Whilst there was a BMI correlation with CSF leptin in men but not in women (see above), an inverse correlation of the CSF/Serum ratio with BMI in women (r=-0.81, p=0.001) was noted, which did not show a correlation with BMI in men (r=-0.17, NS) (Figure 3.3.5.2.A). This means that with increasing BMI the relative or proportional quantity of leptin in CSF in women is lower. This CSF/Serum BMI correlation was previously reported and gave rise to the hypothesis of leptin resistance (Wiedenhoft, 1999).

3.3.3. Adiponectin

3.3.3.1. Serum adiponectin

Serum adiponectin was significantly higher in women (31.5±1.2μg/mL versus men: 22.2±1.1μg/mL (mean±SD), p=0.016). Adiponectin correlated positively with age in men (r=0.56, p=0.01), but not significantly in women (r=0.36, p=NS). Serum adiponectin showed a negative association with BMI in men (r=-0.46, p=0.04), whilst only a trend was noted in women (r=-0.27, NS). In agreement with previous studies, the HOMA-IR index and serum adiponectin were inversely correlated for the total study population (r=-0.48, p<0.001) and the levels significantly lower in the T2DM and insulin resistant subjects than in non-diabetic subjects (19.2±3μg/mL, n=9 versus 28.8±2.9μg/mL, n=30; p=0.02).

3.3.3.2. CSF adiponectin

Mean CSF adiponectin was 16.3±6ng/mL with a range of 7.2-41.6ng/mL, thus the CSF levels were approximately 1000 fold lower than the serum adiponectin levels (Figure 3.3.1). Unlike in the serum, CSF adiponectin did not show a sexual dimorphism (men: 16.7±8.3ng/mL; women: 15.9±3.6ng/mL; p=0.7). However, there was a positive age correlation (r=0.36, p=0.036, n=35) overall which was present in both men and women (r=0.55, p=0.035; r=0.5, p=0.036; respectively). CSF adiponectin was inversely correlated with BMI in men (r=-0.62, p=0.01) but not in women (r=-0.26, NS). HOMA-IR and CSF adiponectin levels showed no correlation or difference between the T2DM and non-diabetic subjects.

3.3.3.3. CSF/serum ratio of adiponectin

The CSF adiponectin levels exhibited a positive linear correlation with serum levels (r=0.34, p=0.047, n=34). CSF/serum adiponectin ratios were significantly higher in men (1.0±0.11 ($*10^{-3}$) than in women 0.65±0.06 ($*10^{-3}$) respectively, p<0.01) and inversely related with serum adiponectin levels (r=-0.72, p<0.001, n=34), suggesting a higher entry of adiponectin into the CSF at low serum values. The best description of this association was achieved by a logarithmic curve fit (Figure 3.3.4 B). The CSF/Serum ratio was inversely correlated with BMI in women (r=-0.61, p=0.02) and did not show a correlation in men (r=0.026, NS) (Figure 3.3.5 B).

3.3.4 Resistin

3.3.4.1. Serum resistin

Mean serum resistin levels were 14.8±8ng/mL and showed slightly higher levels in women (15.8ng/mL versus men: 14.97ng/mL) but this was not significant (t-test p=0.08). Resistin levels correlated positively with increasing age (r=0.45, p<0.01) with no correlation observed with BMI (r=-0.21, p=NS) or HOMA-IR (r=-0.32, p=NS) index.

3.3.4.2. CSF resistin

The mean CSF resistin level was measured as 0.2±0.13ng/mL, with a range of 0.01-0.63ng/mL. CSF resistin levels were not influenced by gender (p=0.65). Furthermore, CSF resistin levels did not correlate with age (r=0.09, NS) and remained unaltered by diabetic status with no correlation with the HOMA-IR index (r=0.24, NS).

3.3.4.3. CSF/ Serum ratio of resistin

The CSF/serum ratio did not differ significantly between males and females. However the CSF/serum ratio correlated strongly with the serum levels in men (r=-0.57, p-0.013) and women (r=-0.77, p=0.001) as best described by a logarithmic graph (Figure 3.3.5.1.B) similar to the correlation described for leptin and adiponectin. The CSF/serum ratio bore no relationship with BMI in either men or women (Figure 3.3.5.2.B).

Variable	All Subjects	men	women
Number of subjects	39	20	19
Age (years)	69.4±9	69.8±8.6	69.4±4.3
BMI (kg/m^2)	28±4.6	29.4±3.4	27.3±4.8
Insulin (mU/L)	5.5±3.4	6.2±4	4.8±2.6
HOMA-IR	1.34±1	1.5±1.2	1.2±0.8
Leptin			
Serum(ng/mL)	17.4±12.5	13±11	22.5±13.7*
CSF(pg/mL)	67±13	56±9	79±9
Adiponectin			
Serum(μg/mL)	26.6±12	22.2±11	31.5±12*
CSF(ng/mL)	16.3±6	16.7±8.3	15.9±3.6
Resistin			
Serum(ng/mL)	14.8±8	14.1±7	15±8
CSF(ng/mL)	0.2±0.13	0.2±0.15	0.19±0.12

Table 3.3.4.: Baseline characteristics of the CSF study population and distribution of adipokines levels (mean±SD) between men and women. Significant gender differences are expressed as * $p<0.05$. SD: standard deviation, BMI: body mass index, HOMA-IR: Homeostasis Assessment Model for Insulin Resistance, CSF: Cerebrospinal fluid

3.3.5. Mode of CSF entry of the three adipokines leptin, adiponectin and resistin

The CSF/serum ratio at a given serum level allows conclusion as to the pattern of CSF entry, *e.g.*, a high CSF/serum ratio suggests that a relatively higher amount is able to enter the CSF and the transport across the BBB is facilitated. If the ratio remained the same, passive diffusion of the protein was more likely.

3.3.5.1. CSF/serum ratio and its relationship with serum levels

The inverse logarithmic curve fit of the CSF/serum ratio with corresponding serum levels for all three adipokines suggests that at low serum levels, there is a higher proportion of protein entering the CSF, which implies a saturated transport mechanism.

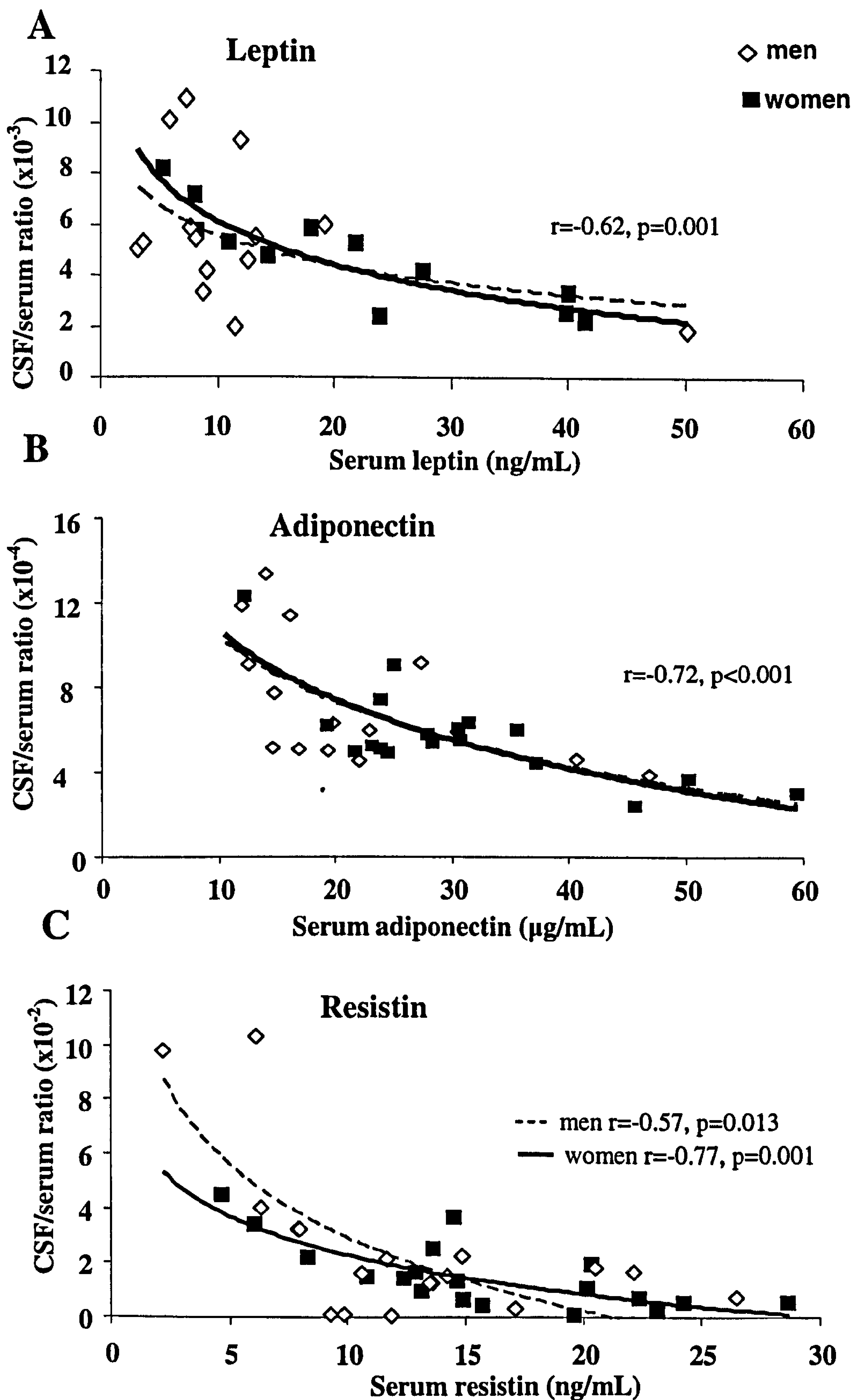


Figure 3.3.5.1.: Association of the CSF/serum ratio with serum levels as logarithmic graphs for **A:** leptin (inverse correlation) **B:** adiponectin (inverse correlation) **C:** resistin (inverse correlation) (r represents the correlation coefficient of curve of best fit, p values as shown on Figure).

3.3.5.2. Comparison of the CSF/serum ratios in relation to BMI

Following figure shows the relationship the CSF/serum ratios with BMI for the three adipokines: leptin, adiponectin and resistin. Whilst neither men nor women show a BMI association with resistin, the curve fit in women describes a significant inverse correlation for leptin and adiponectin as shown in Figure 3.3.5.2..

3.3.5.3. Relationship of the adipokines with insulin resistance

As mentioned above, diabetic patients were not included in the calculation of HOMA-IR, since they were treated with insulin/dextrose infusion before obtaining the blood test. The data shown below therefore exclusively relate to a range of insulin resistance without considering the diabetic range. A comparison of serum and CSF HOMA correlation of all three adipokines shows that there is no correlation of CSF levels of the investigated adipokines with HOMA-IR

3.3.5.3.1. Leptin

As shown above, the leptin levels correlate positively with T2DM. The HOMA-IR or logHOMA-IR were not normally distributed and we therefore used the Spearman coefficient for statistical analysis. Serum leptin was positively correlated with HOMA-IR ($r= 0.431$, $p<0.01$), similar to that observed by previous studies (Silha, 2003). However, both leptin and insulin resistance are also affected by a positive BMI, correlation which may be responsible for this result (Figure 3.3.5.3.1.).

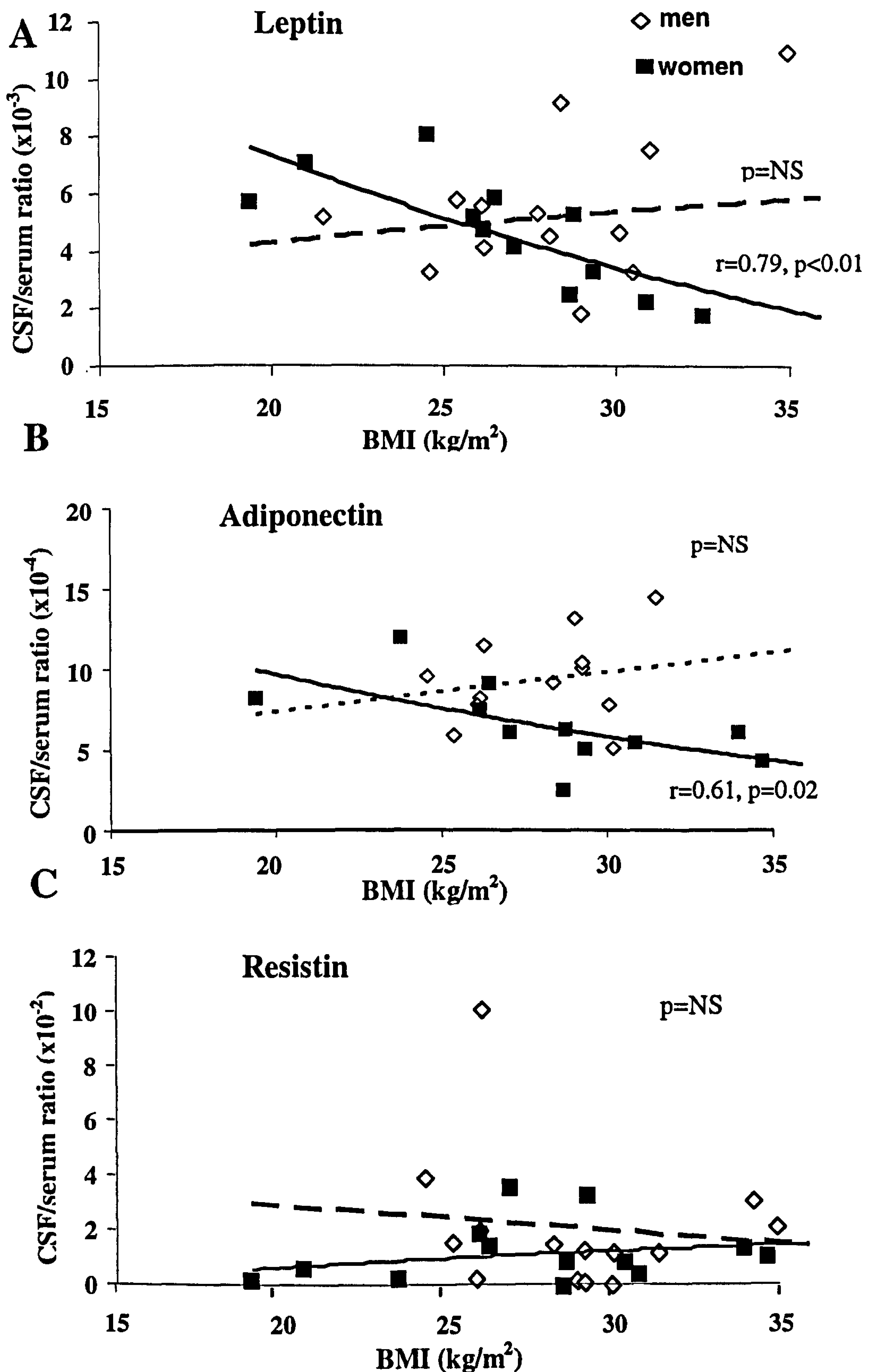


Figure 3.3.5.2.: CSF/serum ratio versus BMI (kg/m^2) for men and women **A:** leptin (inverse correlation in women), **B:** adiponectin (inverse correlation in women) and **C:** resistin (no significant correlation in men or women) (r represents Pearson correlation, p value as shown on Figure).

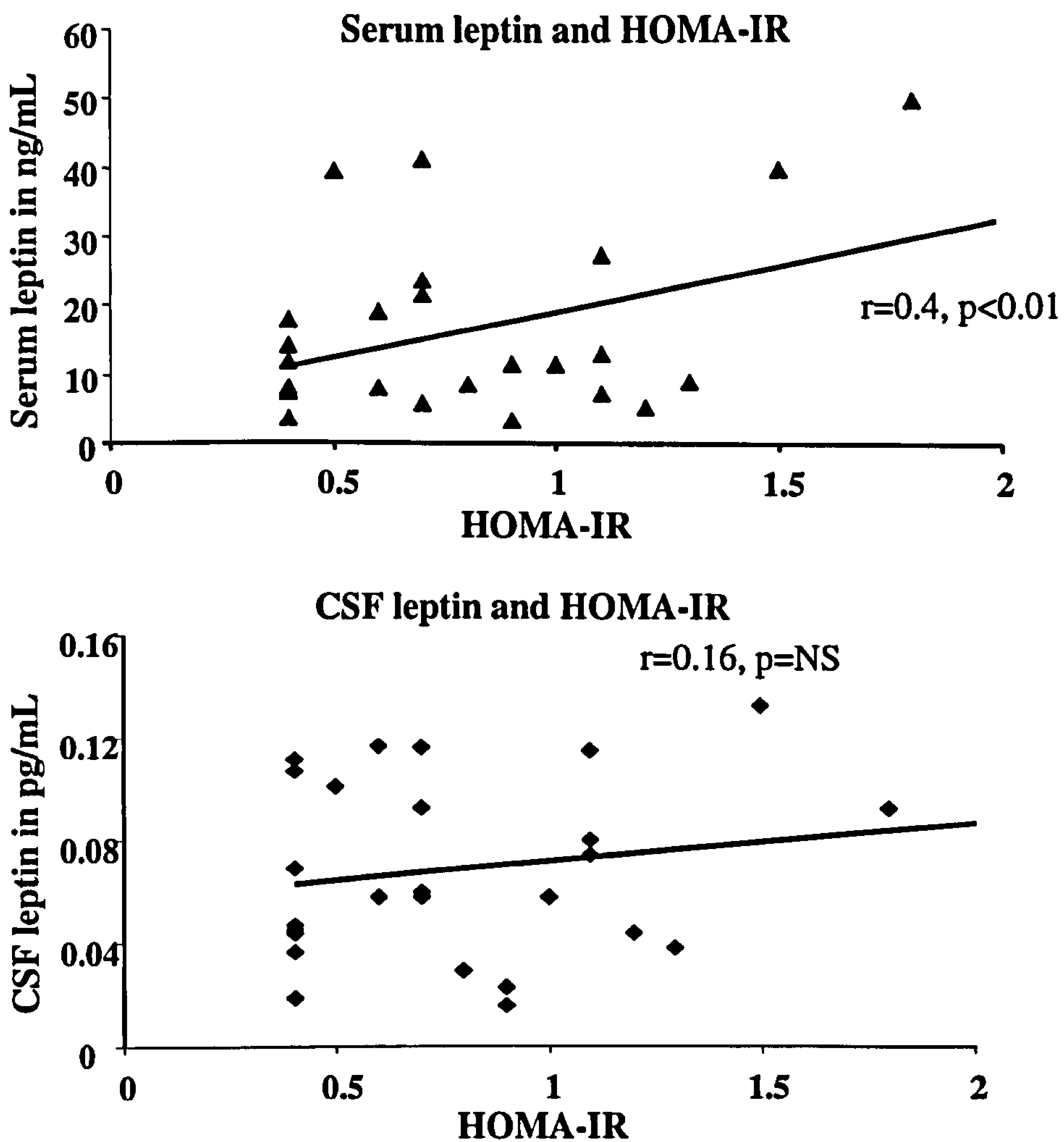
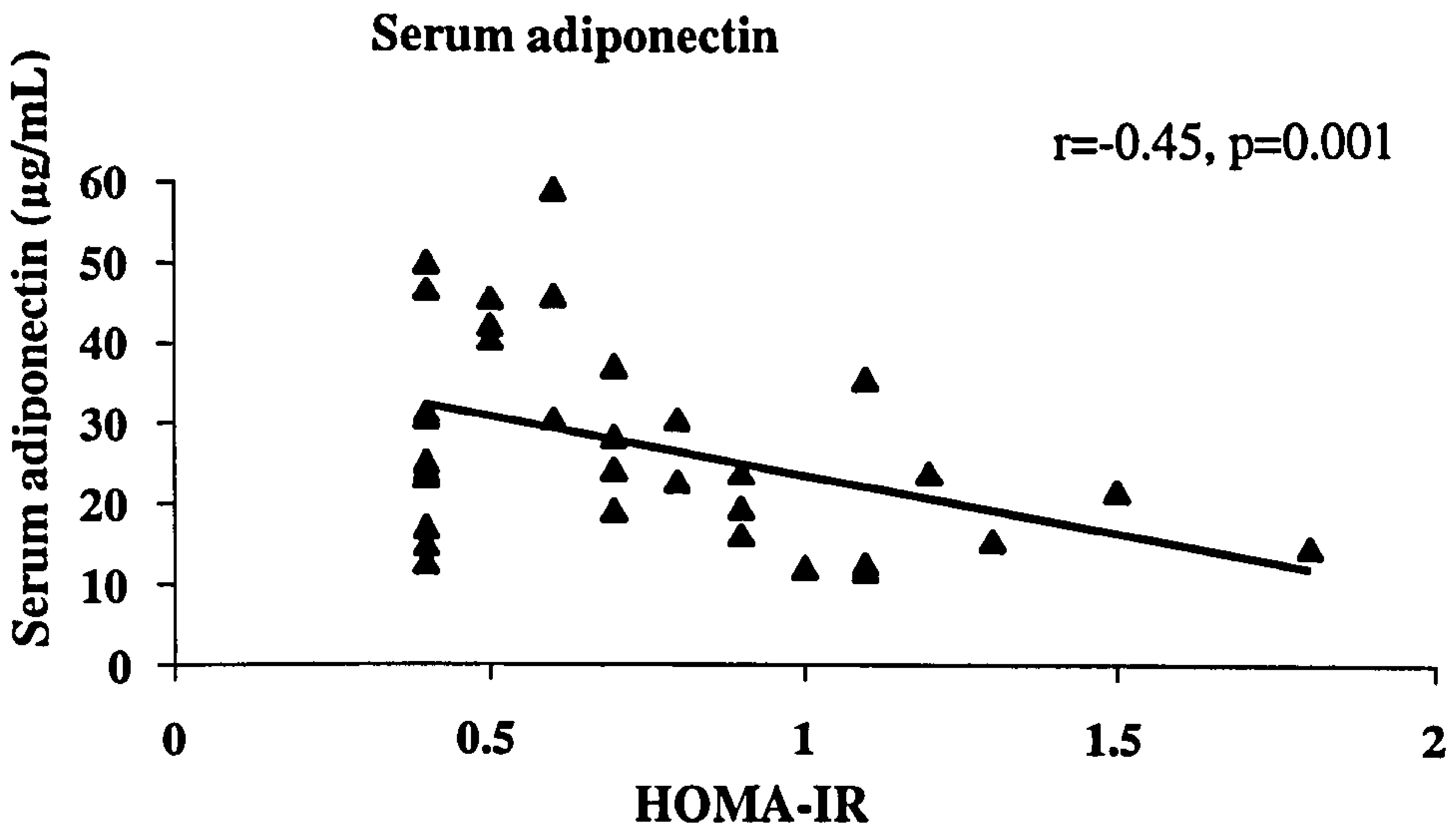


Figure 3.3.5.3.1.: Association of serum and CSF leptin levels with the HOMA-IR. A p value of < 0.5 is considered as significant.

3.3.5.3.2. Adiponectin

There is an inverse correlation of serum adiponectin with HOMA-IR, which is manifest in both men and women (not shown). In CSF, however, there is no correlation of adiponectin levels with insulin resistance. Similarly, there is also no correlation with CSF resistin and the index for insulin resistance.



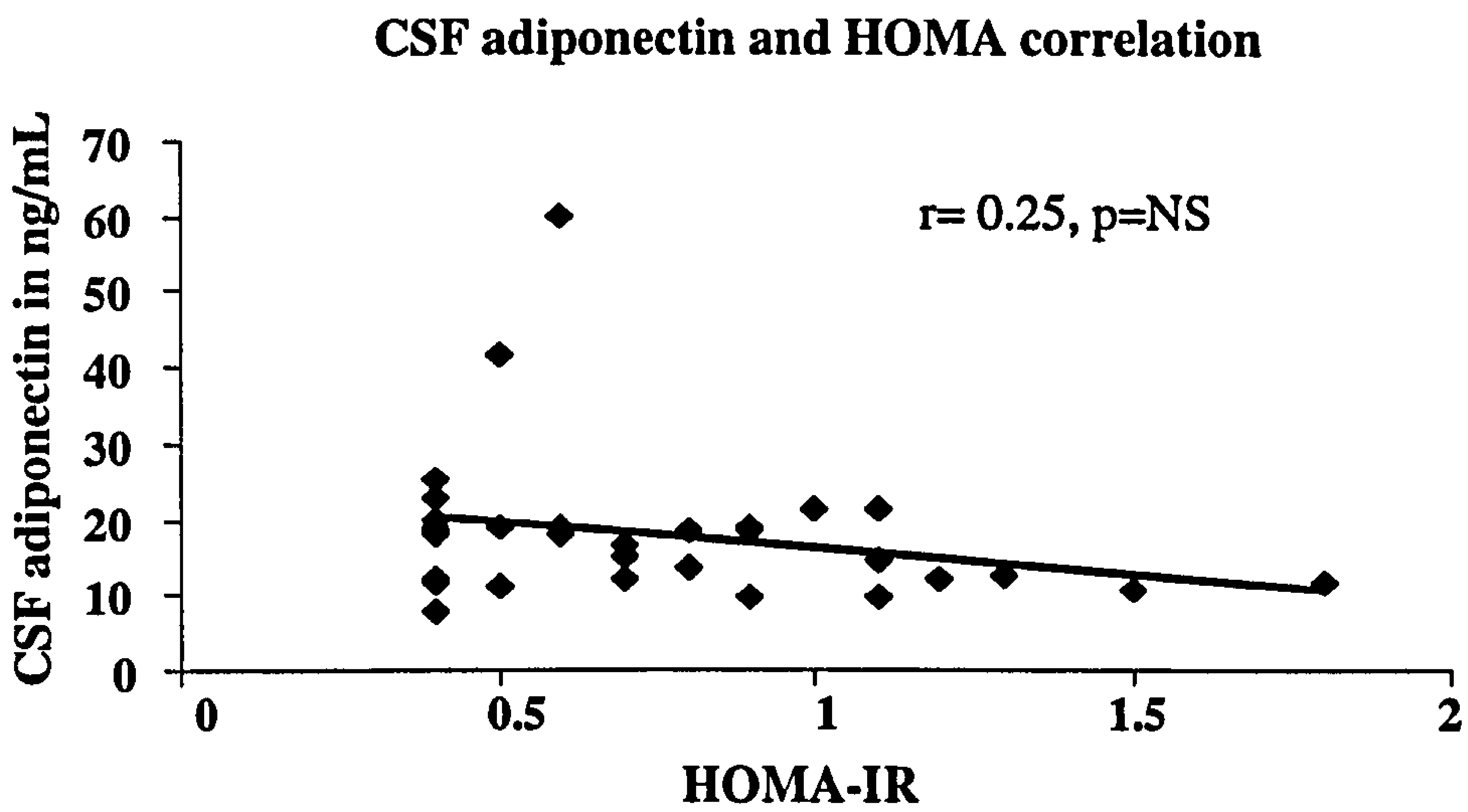
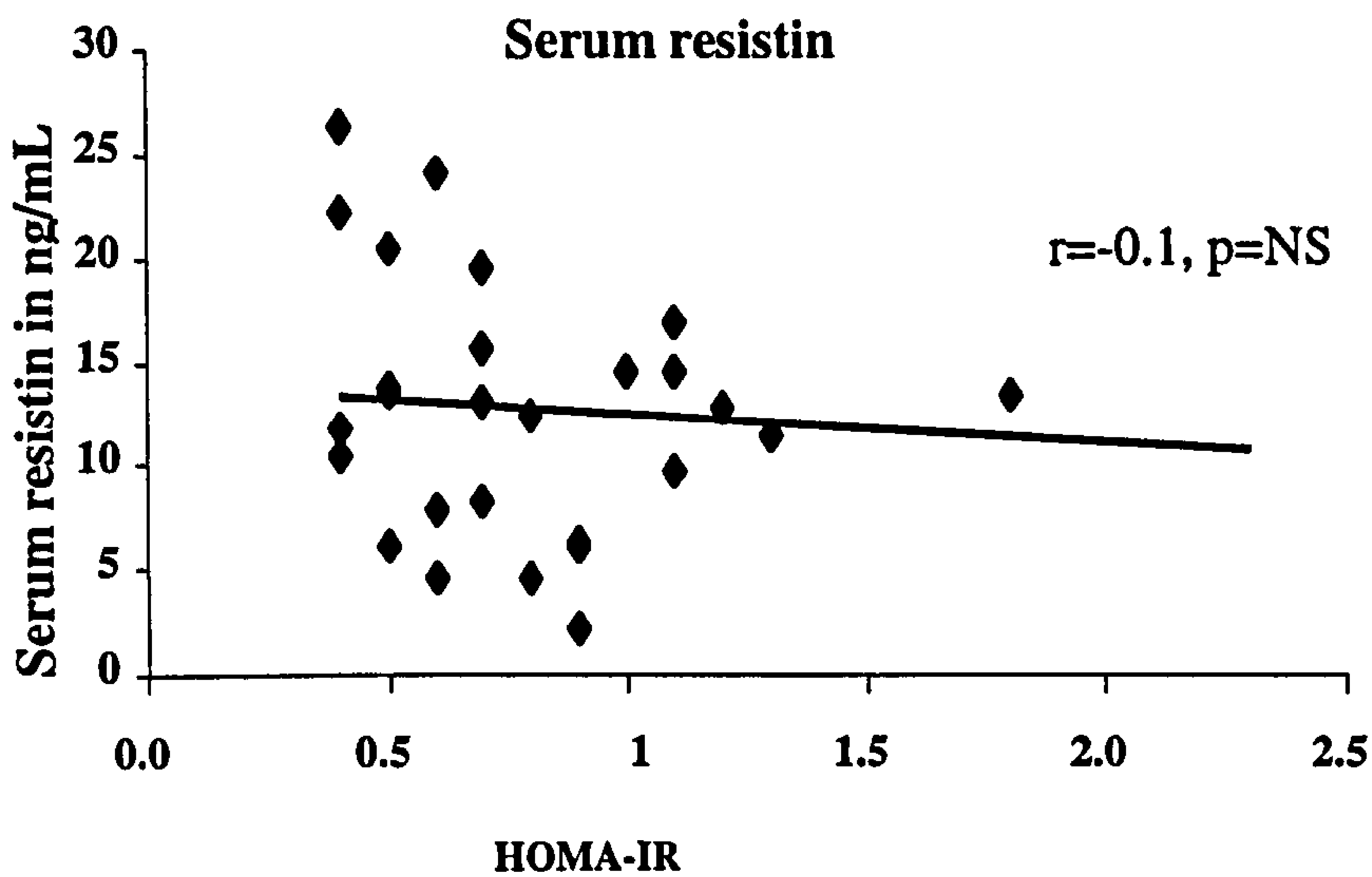


Figure 3.3.5.3.1: Association of serum and CSF adiponectin levels with the HOMA-IR.

3.3.5.3.3. Resistin

Resistin is not correlated with HOMA2-IR within our non-diabetic study population as shown in Figure 3.3.5.3.3.



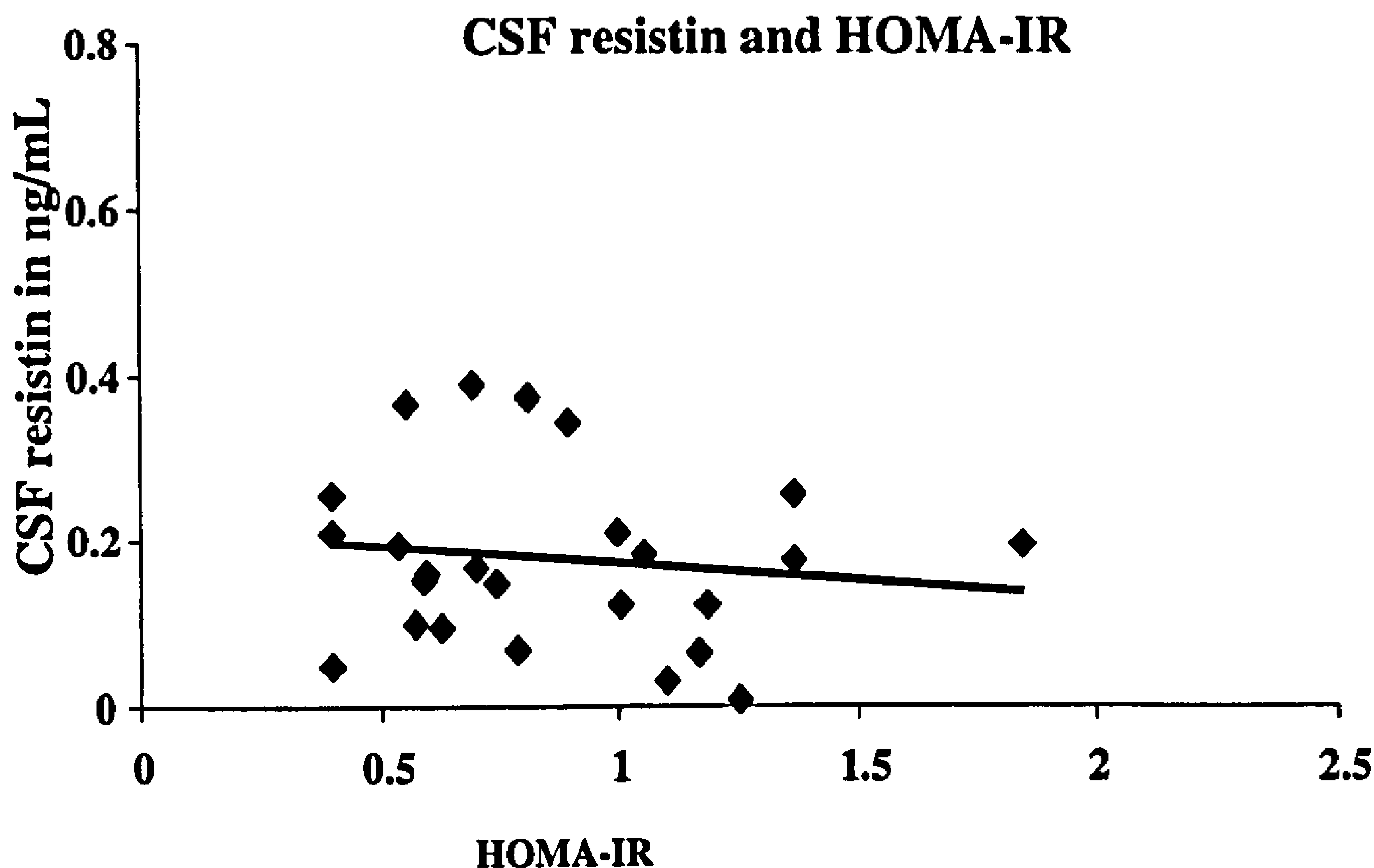


Figure 3.3.5.3.3.: Association of serum and CSF resistin levels with the HOMA-IR in non-diabetic patients.

3.3.6. Adiponectin receptors in the human hypothalamus

Immunohistochemistry showed the expression of AdipoR1 and 2 in the neuronal cells in the human hypothalamus (Figure 3.3.6.). AdipoR1 was expressed in both the anterior and posterior hypothalamus in a diffuse distribution, whilst AdipoR2 was concentrated in the PVN along with neurons which were dual stained with NPY (brown colour).

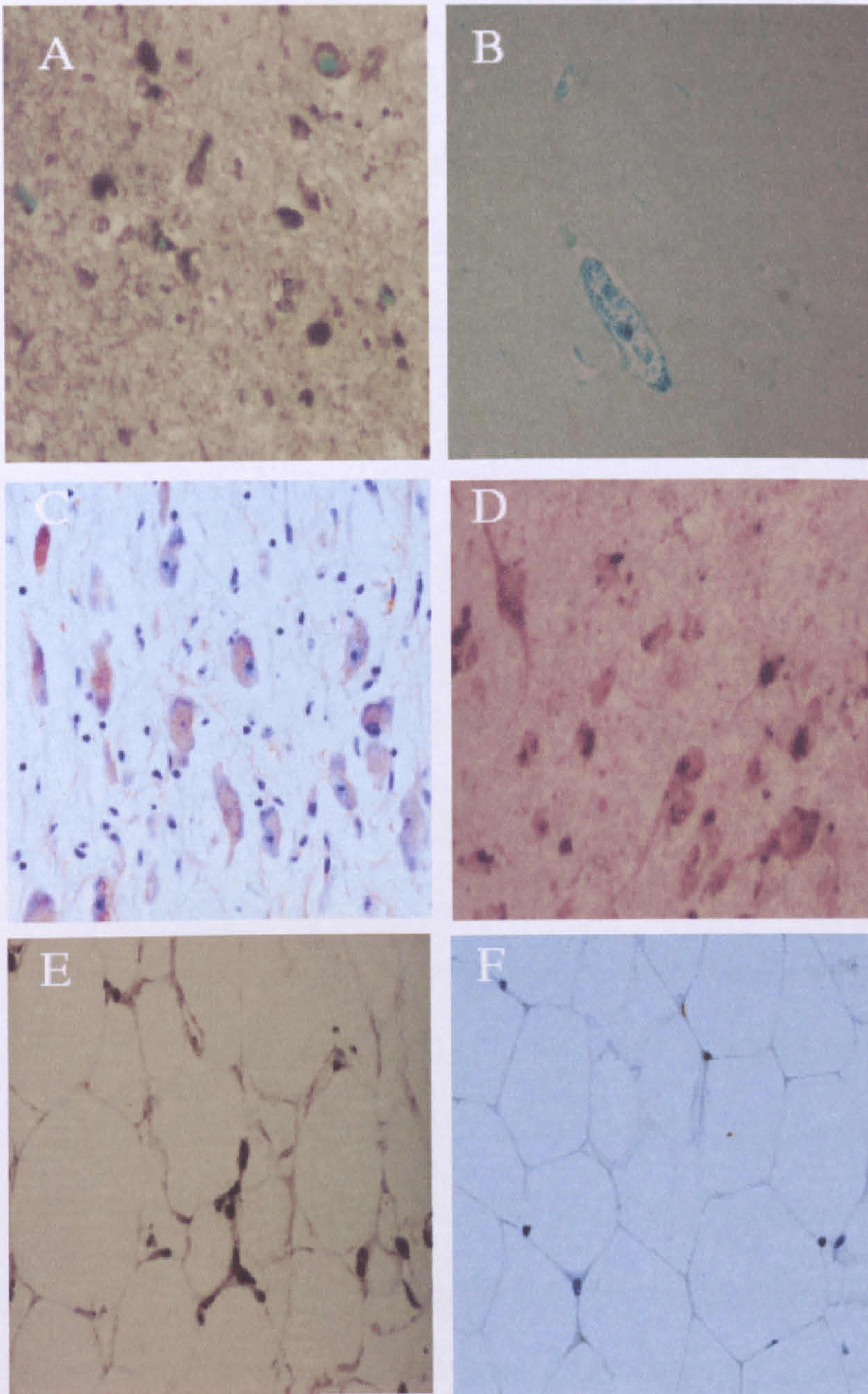


Figure 3.3.6: Immunohistological staining of (A) human hypothalamus with positive staining for AdipR1 (brown; x1000 Magnification); (B) negative staining for human hypothalamus (blue); (C) human hypothalamus with positive staining for AdipR2 (brown; x1000 Mag.) which highlights the area of the paraventricular nucleus and (D) shows dual staining of AdipR2 (red) and NPY (brown); note the dendritic projections of the neural cells and cytoplasmatic expression of AdipR2; (E) human adipose tissue with positive staining for AdipR1(red), (F) negative staining for human AT (blue; x2000 Mag.).

3.4. Discussion

This is the first study to detect the adipokines, adiponectin and resistin, in human CSF. Unlike the recently published findings by Spranger (2006), low adiponectin concentrations were detected which are on average approximately 1000 fold lower, in the CSF than serum. The same 1:1000 ratio of CSF to serum adiponectin was also previously found in rats (Caja, 2005a), but a higher ratio (1:100) has been observed in mice (Qi, 2004). Taken together, these results suggest the presence of adiponectin in CSF in more than one species and a probable species-specific variation. Determination of resistin in human CSF found resistin at approximately levels 100 times lower in CSF than serum. In contrast, no resistin was identified in the CSF of rodents (Caja, 2005b), though resistin mRNA expression was previously found at low levels in the hypothalamus of the mouse brain (Morash, 2002). The relationship between CSF and serum levels varied between patients depending on variables including BMI and the magnitude of the serum levels of the adipokine which, if high, could exceed the threshold of saturation in CSF. This is also the first study to identify presence of AdipoR1 and AdipoR2 not only in the rat brain (Qi, 2004) but in the human hypothalamus, with concentrated expression of AdipoR2 in neuronal cells of the PVN which also express NPY. A previous study has demonstrated mRNA expression of AdipoR1 and 2 on human endothelial cells of the choroid plexus (Spranger, 2006) which controls entry of proteins through the BBB and where functional leptin receptors have also been identified (Merino, 2006). Adiponectin could thus be transported from blood to CSF by receptor mediated transcytosis similar to what was proposed for leptin. A very recent publication (Fry, 2006) not only confirms that AdipoR1 and 2 are expressed in the area postrema of the rat brain, but their

expression mediates adiponectin signals by electrophysiological stimulation, which further supports a central role for adiponectin. These data suggest that adiponectin enters the CSF with potential action via neuronal pathways in the PVN, a hypothalamic area which receives input from the arcuate nucleus – a region of the brain containing key appetite regulating pathways via NPY neurons (Cowley, 1999). Qi and co-workers (2004) have shown increased cFOS immunoreactivity especially in the PVN after intravenous and intracerebroventricular (icv) adiponectin injection into mice, which further strengthens the hypothesis that adiponectin acts in neuronal pathways of the PVN. It is possible that adiponectin's function is dependent on different neuronal networks to leptin, for which studies have highlighted that cFOS immunoreactivity was highest in the arcuate nucleus (Qi, 2004). Nevertheless, the same study provides some evidence of synergistic function, in that both hormones influence the expression of hypothalamic corticotropin-releasing hormone (CRH) and that both use the melanocortin pathway (Qi, 2004). Despite numerous studies, it is still unclear how the adipokine leptin gains access to the arcuate nucleus (Bjorbaek, 1998) and further studies are necessary to identify this and the mechanism of entry into the CNS for adiponectin.

While there is some confirmation of the presence of adiponectin in CSF, receptor expression in the brain and c-Fos activity triggered by adiponectin (Qi, 2004), other studies have failed to establish the presence of adiponectin in human CSF (Spranger, 2006) or could not verify passage of adiponectin through the BBB (Pan, 2006). This may be due to the need for high sensitivity assays to be utilized for its detection. Also, entry of additional adiponectin when administered as radioactive labelled intravenous adiponectin would not show observable

difference due to the low and potentially already saturated levels of CSF adiponectin.

Leptin is a known potent central appetite regulator (Schwartz, 1996b) and its serum levels directly correlate with fat mass (Zhang, 1994). The distribution of leptin levels within this study group was compared for consistency with those previously reported Nam, 2001; Rodrigues, 2002; Wong, 2004; Zloklovic, 2000) and has highlighted variation (Nam, 2001). The average leptin CSF levels in the above findings were relatively low in comparison with some previously reported data (Nam, 2001) whilst similar to other studies (Zhang, 1994; Wong, 2004). A comparison of CSF concentrations of fasting CSF leptin levels (mean 0.04ng/mL, CSF/serum ratio of 1:100) showed that they were much smaller than those of either adiponectin (16.3ng/mL) or resistin (0.2ng/mL). This suggests that the concentrations of CSF adiponectin and resistin reside in the appropriate range to activate CNS effects (Corp, 1998; Zloklovic, 2000).

AdipoR1 and 2 can be activated by both globular and full-length adiponectin. AdipoR1 shows a high affinity for globular adiponectin but data for binding affinities only exist for the muscle receptors in mice (Yamauchi, 2003) and not for neuronal cells in humans. Much lower concentrations than in the periphery are sufficient for activation of neural pathways and signalling in the hypothalamic nuclei for proteins like leptin (Campfield, 1995; Zloklovic, 2000). Adiponectin receptors mediate action through signalling molecules such as PPAR-x, MAPK and AMPK (Tomas, 2002; Fisher, 2005). Serum adiponectin exists in various isoforms, from truncated globular structures to high order molecules. HMW isoforms are attributed with more protective properties than LMW forms (Fisher,

2005). AMPK can be activated by globular adiponectin as well as trimers (Tomas, 2002) and plays a critical role in the central nervous system. Its pathways are involved in hormonal and nutrient signalling in the hypothalamus, including the regulation of food intake by leptin and insulin (Minokoshi, 2004). Besides AdipoR1 and AdipoR2, hexamers and HMW isoforms of adiponectin can also bind to T-cadherin (Hug, 2004) which has yet undefined biological functions. T-cadherin was first discovered in the central nervous system, but is also present in endothelial cells and smooth muscle cells. T-cadherin is unlikely to play a role in central appetite regulation given that HMW do not induce AMPK activation but mediate their action through nuclear factor-κB (NF-κB) in the periphery (Tsao, 2003).

In concordance with previous observations, serum adiponectin is gender dependent, with females showing significantly higher circulating levels (Okamoto, 2000). As in other studies, serum and CSF adiponectin were inversely correlated with BMI in men but not in women (Vilarassa, 2005). Whilst the serum adiponectin levels showed sexual dimorphism, intrathecal adiponectin levels showed no gender difference. This pattern of higher circulating serum levels in women with no CSF gender difference has previously been described for leptin (Wiedenhoft, 1999) and reaffirmed within this study population. A possible gender difference in transport of the BBB in regards of rate and capacity has previously been discussed (Blum, 1997).

This study does not allow determining which isoforms of adiponectin enter the CSF directly. However, with the report of higher serum HMW in women

(Okamoto, 2000) and lack of gender difference in CSF, it is likely that high molecular structures do not enter the CSF. Additionally, due to the size of HMW structures (> 500kDa) these are unlikely to pass through the BBB and multimerisation within the CSF is unlikely.

The CSF/ serum ratio and serum adiponectin association of leptin and adiponectin follows a similar pattern, thus suggesting, that adiponectin enters the CSF space via an active regulated and saturable transport system in a similar manner to leptin (Wiedenhoft, 1999).

Resistin appears to have a role in obesity associated inflammation and insulin resistance which remains controversial in the human context (Hirosumi, 2002; McTernan, 2003). In this study raised circulating resistin levels do not correspond with higher CSF levels suggesting limited uptake into the CSF. The wide variation in resistin concentration requires further studies to examine entry of resistin across the BBB. No correlation between serum and CSF resistin levels were observed with BMI and neither of the adipokines' CSF levels were noted to be affected by insulin resistance. T2DM is also associated with reduced HMW isoforms (Fisher, 2005) and impairs the multimerisation of adiponectin (Waki, 2003). Thus, adiponectin CSF levels may predominantly constitute of LMW isoforms for reasons explained above and, as such, may not be affected by diabetes.

In summary, adiponectin and resistin are present in human CSF with levels unaffected by gender. Adiponectin receptors AdipoR1 and AdipoR2 are present in the human hypothalamus, especially the PVN which strengthens the hypothesis that they are involved in central energy homeostasis in humans. These findings

highlight the potential involvement of adipokines in central energy homeostasis in humans. Based on this data, an active transport mechanism for both adiponectin and resistin across the BBB is likely.

<u>Chapter 4</u>

NPY and adipose tissue

4.1. Introduction

The neurotransmitter NPY has been well characterised for its central orexigenic function and hypothalamic levels of NPY reflect the nutritional status (Wilding, 1992). However, NPY and its receptors are not only present in the central nervous system, but in many peripheral sites where they are involved amongst others in the regulation of angiogenesis, vasoconstriction, mood regulation and fertility (Chronwall, 2004). Within the periphery, NPY has been identified as a hormone which can promote fat accumulation through upregulation of lipoprotein lipase (LPL) activity, thus supporting an antilipolytic role for NPY (Billington, 1991). Several studies have confirmed the presence of receptors mediating NPY action in adipose tissue (AT) in mammals including humans (Castan, 1994; Labelle, 1997; Bradley, 2005). Currently, the presence of NPY in AT is thought to be derived through the sympathetic nervous system (SNS) innervation of white adipose tissue (WAT), with depot specific differences in rodents and humans (Dodt, 1999). NPY is coreleased with the neurotransmitter norephinephrine (NE) in peripheral, adrenal and central catecholaminergic neurons (Everitt, 1984). NE is a potent stimulator of lipolysis (Carpene, 1998) and an increase in SNS innervation induces increased lipid mobilisation and glycerol release (Dodt, 1999), which is thought to be mediated by NE. NE action and SNS innervation thus contradicts the antilipolytic effect of NPY. Thus for NPY to mediate an opposing effect to its cotransmittter NE, an independent source of NPY free of SNS is possible, *i.e.*, by secretion of NPY from adipocytes.

Insulin and leptin are key regulators of energy homeostasis, as discussed in Chapter 1. Circulating levels of insulin and leptin are directly correlated to the

amount of body fat mass (Considine, 1996; Bagdade, 1967); furthermore, intracerebroventricular (*icv)* administration of insulin and leptin can reduce food intake (Campfield, 1995; Woods, 1979), consistent with crosstalk between the brain and adipose tissue and a central feedback circuit for energy homeostasis. *Icv* administration of insulin and leptin not only decreases food intake, but also reduces the fasting induced rise of NPY mRNA in the arcuate nucleus (Porte, 1981; Sato, 2005; Fekete, 2006). Little, however, is known about a possible interaction of these appetite regulators: NPY, leptin and insulin in the periphery.

Y1 (NPY receptor 1), as one of four NPY human receptors, is thought to mediate the antilipolytic function of NPY (Bradley, 2005). Cleavage of proline by dipeptidyl peptidase-IV (DPP-IV) generates $NPY_{3\text{-}36}$ from $NPY_{1\text{-}36}$ and diverts NPY binding from Y1 to other receptors (Mentlein, 1999). Consequently, DPP-IV can be regarded as an endogenous Y1 receptor antagonist (Zukowska-Grojec, 1998a) to inhibit NPY's antilipolytic activity. It would therefore be of interest to determine whether DPP-IV is present in AT and, how its expression is related to obesity. DPP-IV inhibitors are novel therapeutic agents in the treatment of T2DM (*i.e.*, vildagliptin and sitagliptin) with the purpose to increase the half-life of the incretin hormone glucagon like peptide-1 (GLP-1) (Burkey, 2005). GLP-1 is also cleaved by DPP-IV and DPP-IV reduces its insulin secreting potential (Ahren, 2004). However, the effect of DPP-IV inhibitors on NPY metabolism with $NPY_{1\text{-}36}$ being one of DPP-IV best substrates (Mentlein, 1999) has, to date, received little attention.

The aim of this study was firstly, to determine protein expression of NPY in adipocytes; secondly, to investigate whether NPY is regulated by insulin and leptin and whether its secretion is modulated by the PPARγ agonist, rosiglitazone

(RSG); thirdly, to determine the influence of NPY on the level of adiponectin secretion and finally, to evaluate the expression of DPP-IV in AT, which is known to modulate the function of NPY.

4.2. Materials and Methods

4.2.1. Subjects

AT samples were obtained from thirty eight consenting Caucasian female subjects undergoing elective surgery (mean age: 42.7±7.3years; BMI: 26.2±4.5kg/m^2 (mean±SD)). Abdominal subcutaneous (Abd Sc), omental (Om) and thigh tissue (Th) samples were collected with the approval of the South Birmingham Ethics Committee. Subjects receiving endocrine therapy (e.g., steroids, HRT, thyroxine) and patients with malignant diseases were excluded. A separate cohort of Abd Sc (n=10) AT samples from female patients (age: 45.4±9.2years; BMI: 28.1±2.2kg/m^2) was used for microarray analysis and flash frozen prior to RNA extraction.

Additionally, fasting serum samples were taken from 24 non-smoking Caucasian healthy volunteers (13 men and 11 women) with approval of the Coventry and Warwickshire Ethics Committee. (Mean age: 45.4±9.2years; BMI: 28.0±5.8kg/m^2). Peri- or postmenopausal women were excluded as well as people with history of a recent weight change. Serum was centrifuged at 700 g for 15 min, snap frozen and stored at -80°C to be used for protein analyses as described below.

4.2.2. Adipose tissue processing and tissue culture

The freshly acquired AT was split into two sections. The first part was immediately flash frozen and stored at -80°C to be kept for later protein extraction with RIPA, whilst the remainder was utilised for *in vitro* culture of isolated Abd Sc mature adipocytes. The procedure of adipocyte isolation and cell culture are in detail described in Chapter 2.1. and 2.2. Abd Sc adipocytes were

treated with rh Insulin (1,100 and 1000nM) (human recombinant insulin expressed in *Escherichia coli*; Sigma, Poole, UK), insulin in combination with RSG (10^{-8}mol/L) (GlaxoSmithKline, Harlow, UK) and insulin (100nmol/L) or RSG alone (10^{-8}mol/L) for 48 hr. Untreated adipocytes were used as controls. These media were used to measure NPY secretion by ELISA. Some of the Abd Sc adipocytes were also treated with 1, 10 and 100nmol/L of rh NPY (Sigma-Aldrich, UK) for 48 hr and the conditioned media used for analysis of various adipokines was performed (see below) by ELISA. In parallel, a series of the same NPY treatments was performed with addition of 1M DPP-IV (dipeptidyl esterase-IV blocker (Sigma, Poole, UK).

4.2.3. Protein extraction and NPY Western blot analysis

A small piece of frozen tissue was homogenized and extracted with RIPA buffer (see Chapter 2.3.1.) The protein content was then determined using a Bio-Rad DC protein assay kit (BioRad, Hercules, California, USA). AT protein was loaded onto a 15 % gel with amounts of 20µg/lane and, adipocytes with amounts of 60µg/lane; then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). All samples were then heated for 5 min at 95°C in a sample buffer (Chapter 2.4.). Pre-stained molecular weight markers (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used as standards. Samples were electrophoresed at 140V for 1.5 hr. Proteins were transferred from the polyacrylamide gels to PVDF membranes by electroblotting in a vertical transfer apparatus tank at 100V for 1hr. Membranes were blocked overnight at 4°C in phosphate buffered saline containing Tween 20 (PBS-T: PBS + 0.05% Tween 20; Sigma-Aldrich, UK) containing 10% (w/v) non-fat milk powder

(Marvel, Moreton, Merseyside, UK). Membranes were treated with primary polyclonal goat NPY antibody (Santa Cruz Biotechnology, Inc.) (1:1000), then the membranes developed with a conjugated anti-goat/sheep HRP secondary antibody in concentrations of 1:80,000 in PBS with 0.5% Tween. The prepro-NPY protein (11kDa) and mature NPY (4kDa) were detected by chemiluminescent assay ECL^+ (Amersham, Little Chalfont, UK), which enabled visualisation after exposure to X-ray film and the band intensity was determined by densitometry (Chemigenius). Equal loading was ensured with use of the alpha tubulin antibody (Abcam, Cambridge, UK). To ensure specific binding, a NPY blocker was used, which competes with NPY antibody binding. The blocking peptide (Santa Cruz Biotechnology Inc., USA) was used as recommended by the manufacturer. Essentially the membrane was incubated with the blocking peptide at a dilution of 1:25 together with the primary antibody in PBS at 4°C overnight, and after a three washes in PBS/PBS Tween, the membranes were treated with the secondary antibody as described previously.

Similar to NPY, the expression of the NPY receptors Y1 and Y5 was examined by Western blot analysis. The alterations to the protocol above included loading of 20 μg of protein under reducing conditions onto a 12% gel, which was run for 1 hr and use of monoclonal human Y1 and Y5 antibodies (Bachem Peninsula Laboratories, USA) at concentrations of 1:500 for 1 hr and with the secondary rabbit antibodies at concentrations of 1:60,000 for 1 hr.

4.2.4. NPY and adipokine estimation

Conditioned media (500μL) was concentrated with the use of a centrifuge evaporator and reconstituted in 50μL of assay buffer and assessed for NPY by the commercially available NPY ELISA kit (Bachem Peninsula Laboratories, USA), which was also used for analyses of undiluted serum samples (loading volume of 50μL). The assay sensitivity is reported at 0.04-0.06ng/mL, the intra-assay variation at <5% and inter-assay variation at <14%. Adipokines in serum and media samples were measured by commercial ELISA-based colorimetric kits: For the leptin ELISA (R&D Systems, Inc., Abingdon, UK) serum was diluted 1:100 and cell culture media at 1:10. The assay limit of leptin was 7.8pg/mL, intra-assay limit 3.2%CV and inter-assay variability of 4.4%. Adiponectin in cell media was analysed at a dilution of 1:50 by the ELISA kit from Linco Research Inc. (Missouri, USA); assay limit was 0.78ng/mL intra-assay %CV of 7.4% and inter-assay variability of 2.4-8.4%.

4.2.5. Immunohistochemistry

The histological specimens were prepared as discussed in Chapter 2.7.; incubated with primary polyclonal NPY antibody (Santa Cruz Biotechnology Inc., USA) in a dilution of 1:400 and developed using Vector VIP peroxidase substrate kit (Vector Laboratories Ltd., Peterborough, UK). Rat brain tissue was used as positive control for NPY staining and obtained from Biological Solutions LTD. Slides were prepared and stained as described in Chapter 3. Sections were developed using diaminobenzidine (BioGenex, California, USA). Primary antibody staining was omitted as negative control to exclude contamination and artefacts.

4.2.6. Microarrray analysis

The protocol of microarray analysis is in detailed in Chapter 2.6. and the method described in Appendix II. This technique was used to screen for presence of the chromosome for DPP-IV is chr2q24.3.

4.2.7. Statistical Analysis

Statistics was performed using the SPSS version 14 (SPSS UK, Surrey, UK). For assessment of protein expression and secretion, statistical analysis was undertaken with paired t-test for comparison of Western blots and ANOVA for comparison of cell treatments. Correlations of serum levels were determined with a Pearson correlation. P values of less than 0.05 were considered as statistically significant. Values are expressed as mean +/- standard error (SE) unless otherwise stated.

4.3. Results

4.3.1. NPY protein expression in fat

Protein studies determined that prepro-NPY (11kDa) and mature NPY (4kDa) were detected in AT and isolated adipocytes. The treatment with NPY peptide blocker is illustrated in Figure 4.3.1.

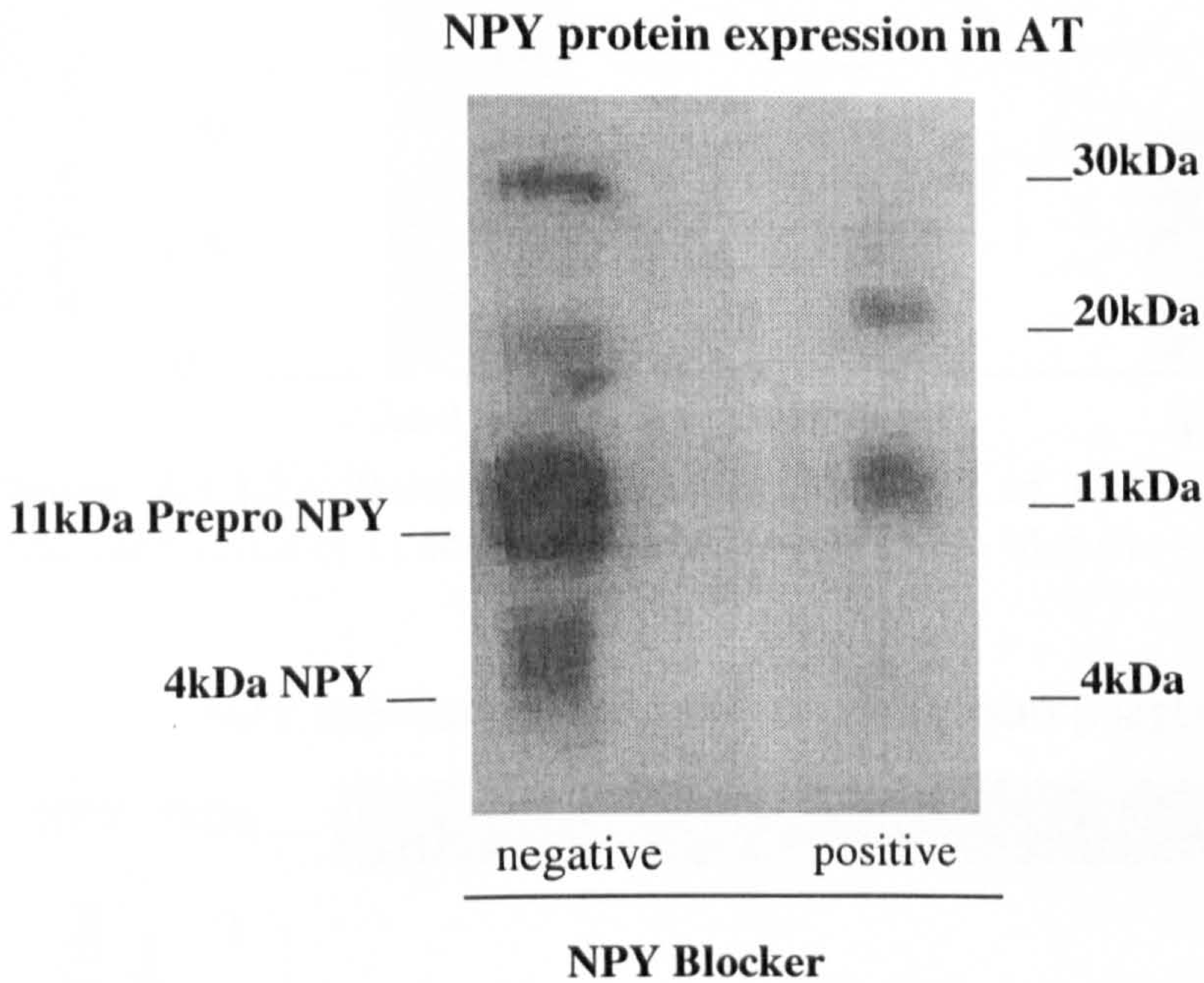


Figure 4.3.1.1.: Expression of NPY and the use of NPY blocker in Abd Sc AT. The left side shows that addition of the NPY blocker effectively blocks the antibody binding of the protein to its antibody.

Western blot analysis was used to examine the depot expression of NPY. NPY was found in Abd Sc approximately with 2 fold higher protein expression than in either omental (Om) or thigh (Th) AT (Abd Sc: 1.87±0.23 optical density units (OD), Om: 1.03±0.15OD, Th: 1.0±0.29OD, p=0.029 and p=0.035 respectively) (Figure 4.3.2).

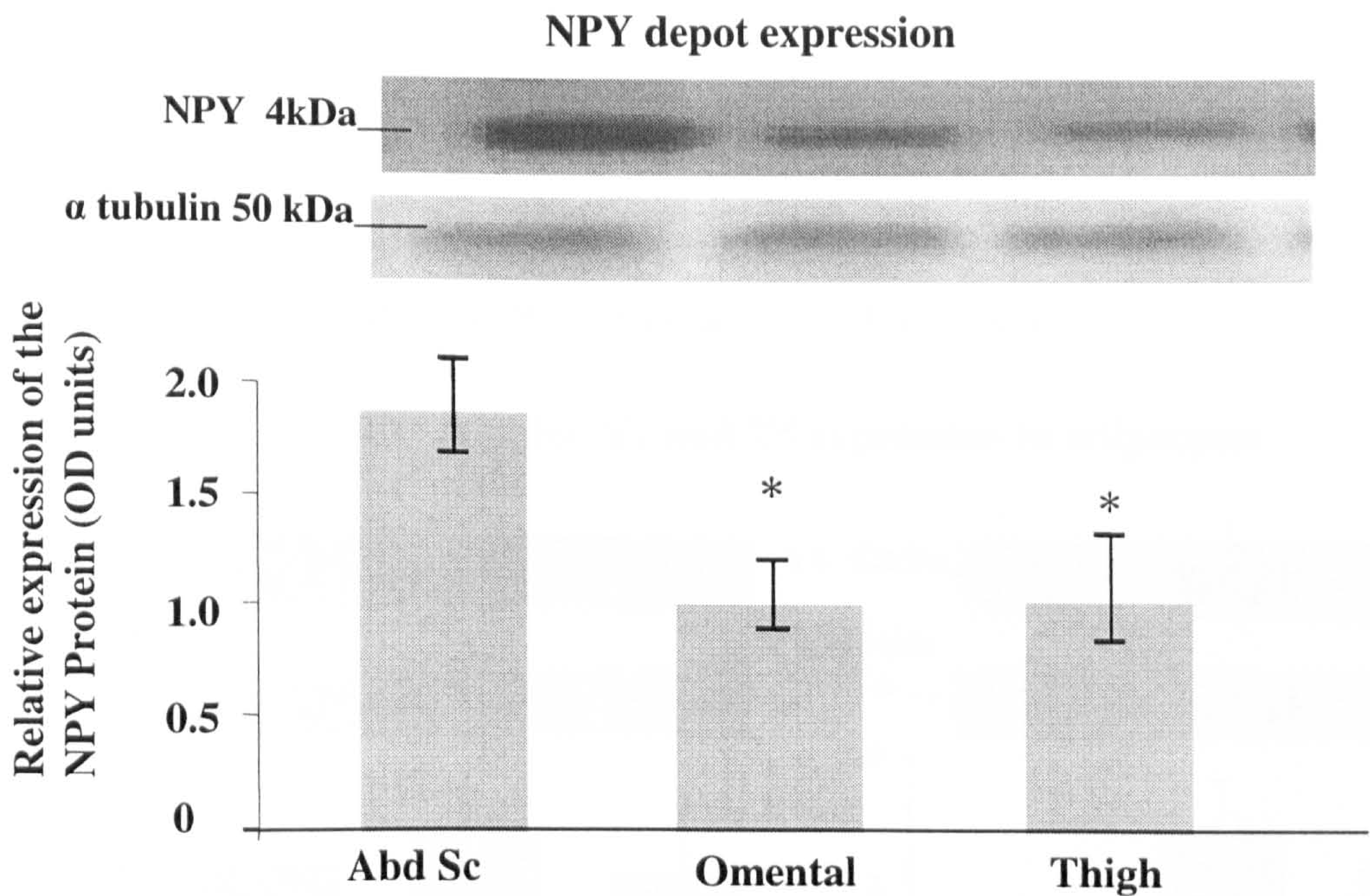


Figure 4.3.1.2.: Relative expression of NPY in comparison to thigh fat (allocated value of 1) and a representative Western blot shown above *p<0.05.

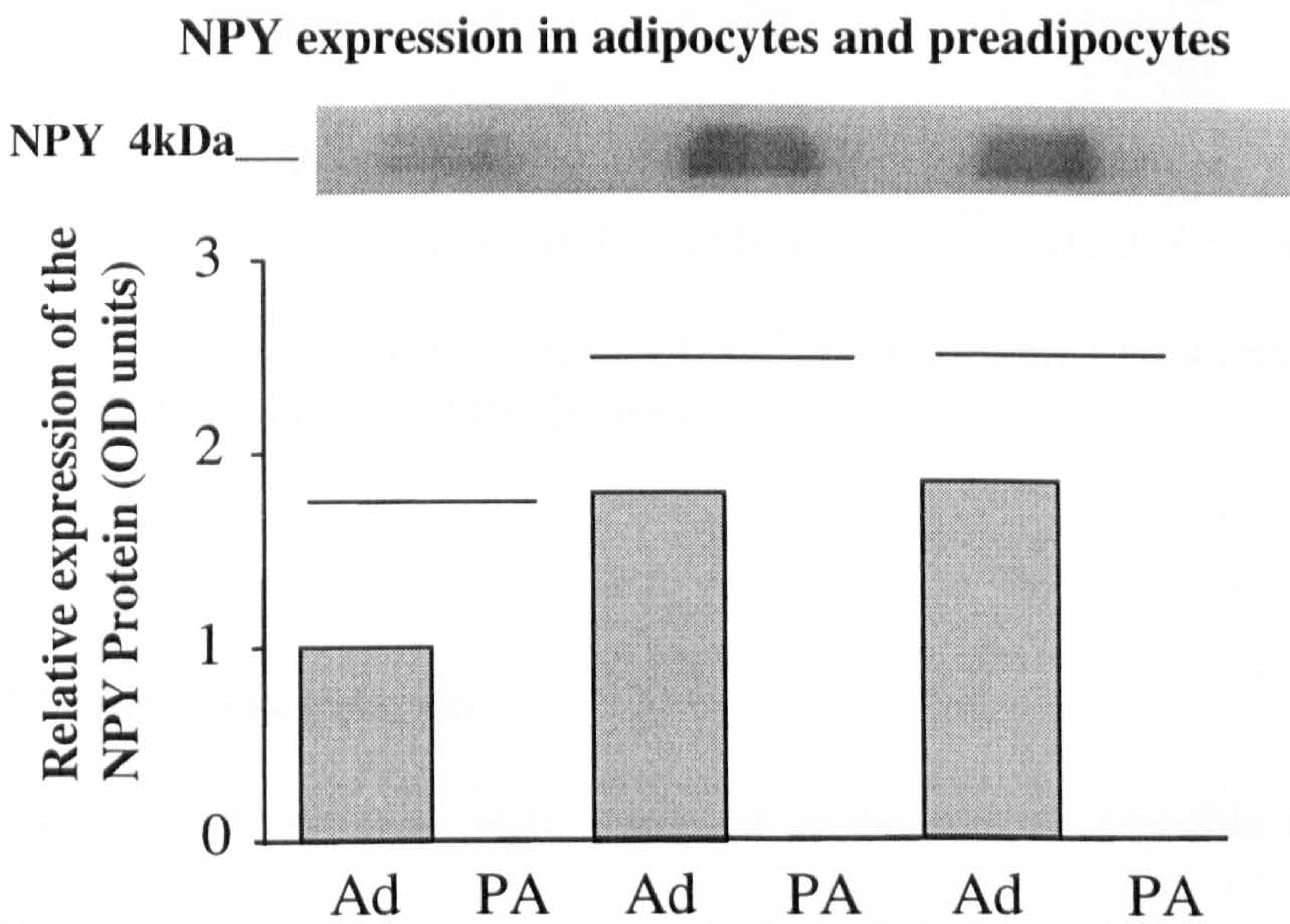


Figure 4.3.1.3.: Expression of NPY in human adipocytes (Ad) but not preadipocytes (PA).

Protein expression is detectable in adipocytes but not preadipocytes as is shown in Figure 4.3.3.. The adipocytes and preadipocytes were derived from the same patient, for each assessment.

The receptors Y1 and Y5 proteins are also found to be expressed in human AT, whilst no depot specific distribution was shown (Y1: AbSc: 2.96±0.13OD, Om: 3.21±0.35OD, Th: 2.95±0.2OD, p=NS, n=6; Y5: AbSc: 2.83±0.1OD, Om: 3.23±0.2OD, Th: 3.04±0.18OD, p=NS, n=6 (see Figure 4.3.4.).

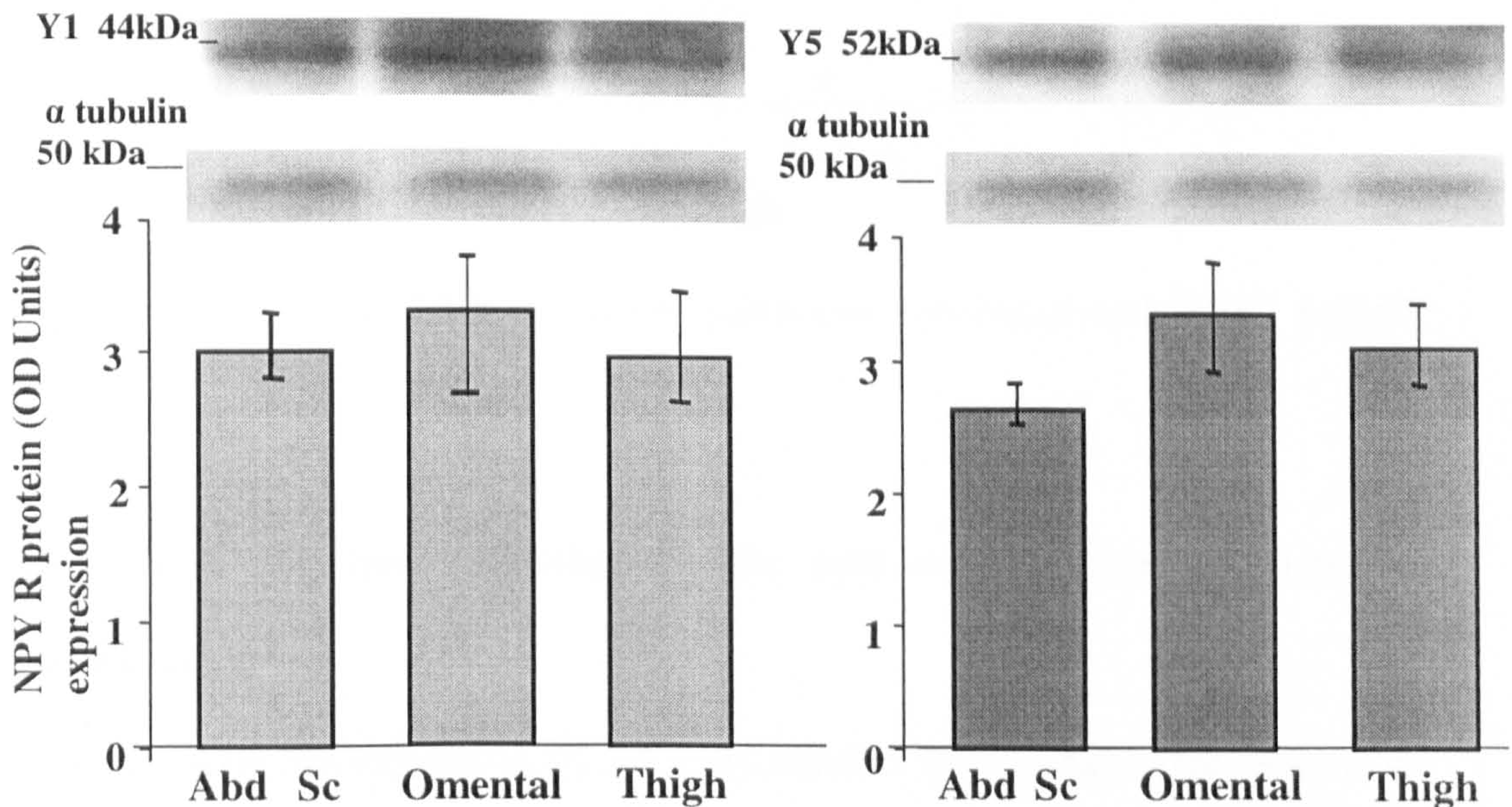


Figure 4.3.1.4.: NPY receptors Y1 and Y5 are expressed in adipose tissue but show no depot-specific distribution.

4.3.2. NPY serum levels

Fasting serum samples were analysed to establish a possible correlation with BMI, gender and age in an age and BMI matched population amongst gender. A positive association was found for NPY serum levels with BMI (r=0.52, p=0.02, n=24) (Figure 4.3.2.). There was no statistical difference between the serum NPY values of men (201±32ng/mL, BMI: 26.4±1.1kg/m^2, n=13) and women (220±35pg/mL, BMI: 24.7±1.2kg/m^2, n=11; p=NS), nor was there a correlation with age (r=-0.12; p=NS).

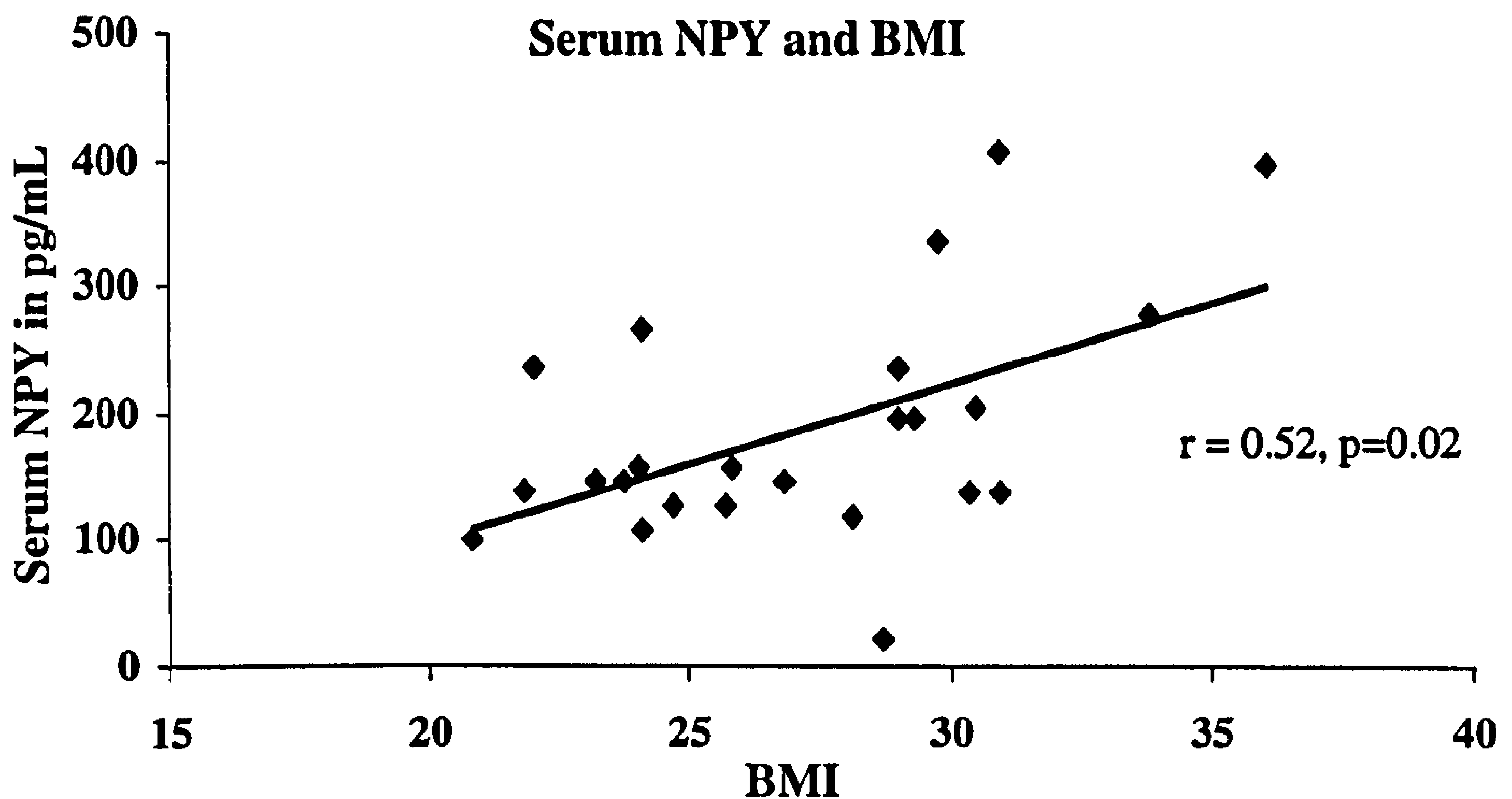


Figure 4.3.2.: Serum NPY levels are positively correlated with BMI, $p<0.05$, n=24.

4.3.3. NPY secretion following insulin and rosiglitazone treatment of adipocytes

To investigate the regulation of NPY by insulin, freshly harvested adipocytes were treated with rh insulin (see above) and changes in NPY secretion measured with ELISA. NPY was significantly up-regulated with insulin treatment (1,100 and 1000nmol/L) (0.26±0.05ng/mL; 0.29±0.04ng/mL; 0.3±0.04ng/mL respectively; control: 0.22±0.02ng/mL, p=0.013, n=13; Figure 4.3.3.1.).

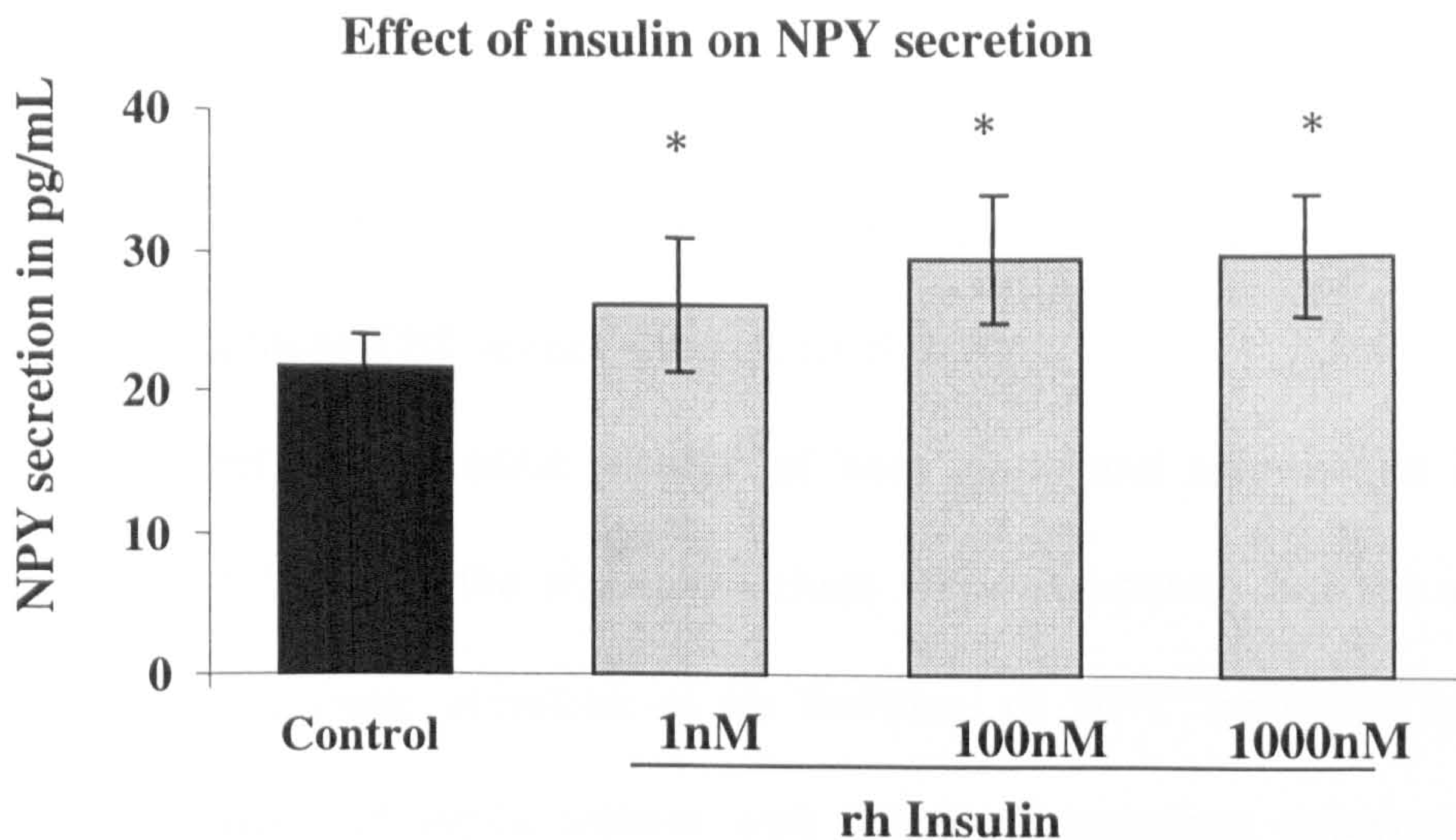


Figure 4.3.3.1.: Effect of rh Insulin on NPY secretion, nM= nmol/L, n=13; *p<0.05.

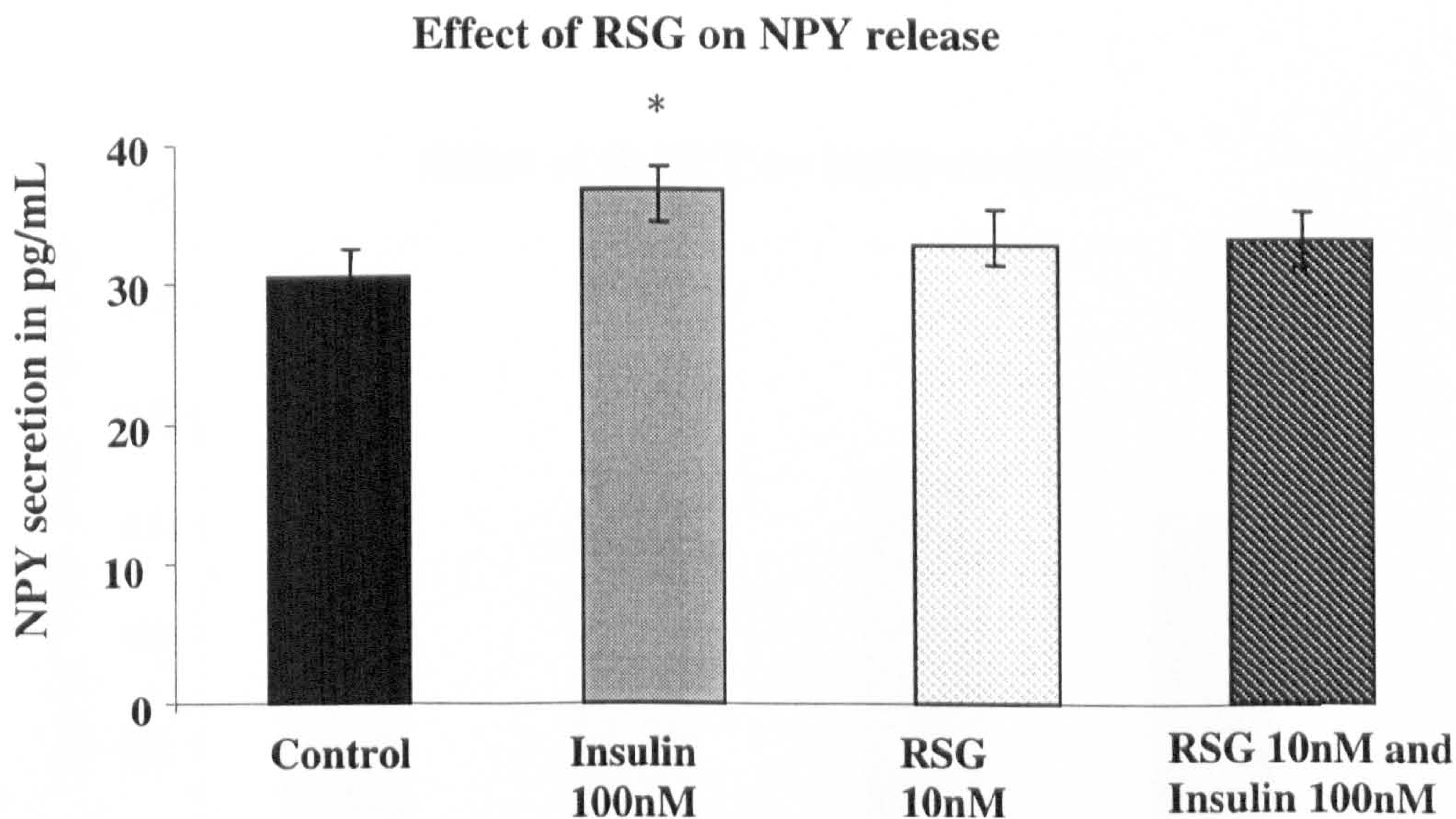


Figure 4.3.3.2.: The effect of RSG and insulin treatment on the level of NPY secretion from cultured adipocytes, n=7, *p<0.01

NPY secretion from adipocytes did not differ whether the adipocytes from the same subject were treated with insulin 100nmol/L, insulin 100nmol/L in combination with RSG 10 nmol/L, or RSG alone (insulin alone 100nmol/L: 36±5ng/mL versus insulin in combination with RSG: 33±4ng/mL or RSG alone 33±4ng/mL, p=NS, n=7; Figure 4.3.3.2.).

4.3.4. Adipokine secretion following rh NPY treatment of adipocytes

Cell culture treatment of adipocytes isolated from female subjects (age=39.3±3.2years; BMI 26.4±2kg/m^2 n=7) revealed a regulation of leptin, but not for adiponectin or TNF-α (not shown), by rh NPY.

The basal secretion (of control sample) of both leptin and adiponectin is BMI dependent, therefore not the absolute values were compared, but the relative changes from the basal secretion as its fraction. rh NPY treatment led to a significant decrease of leptin release with the basal secretion adjusted to the value of 1 the calculated change was 0.620.09; 0.67±0.07 and 0.61±0.08 for treatment with rh NPY 1, 10 and 100nmol/L respectively (n=9, $p<0.05$; Figure 4.3.4.1.).

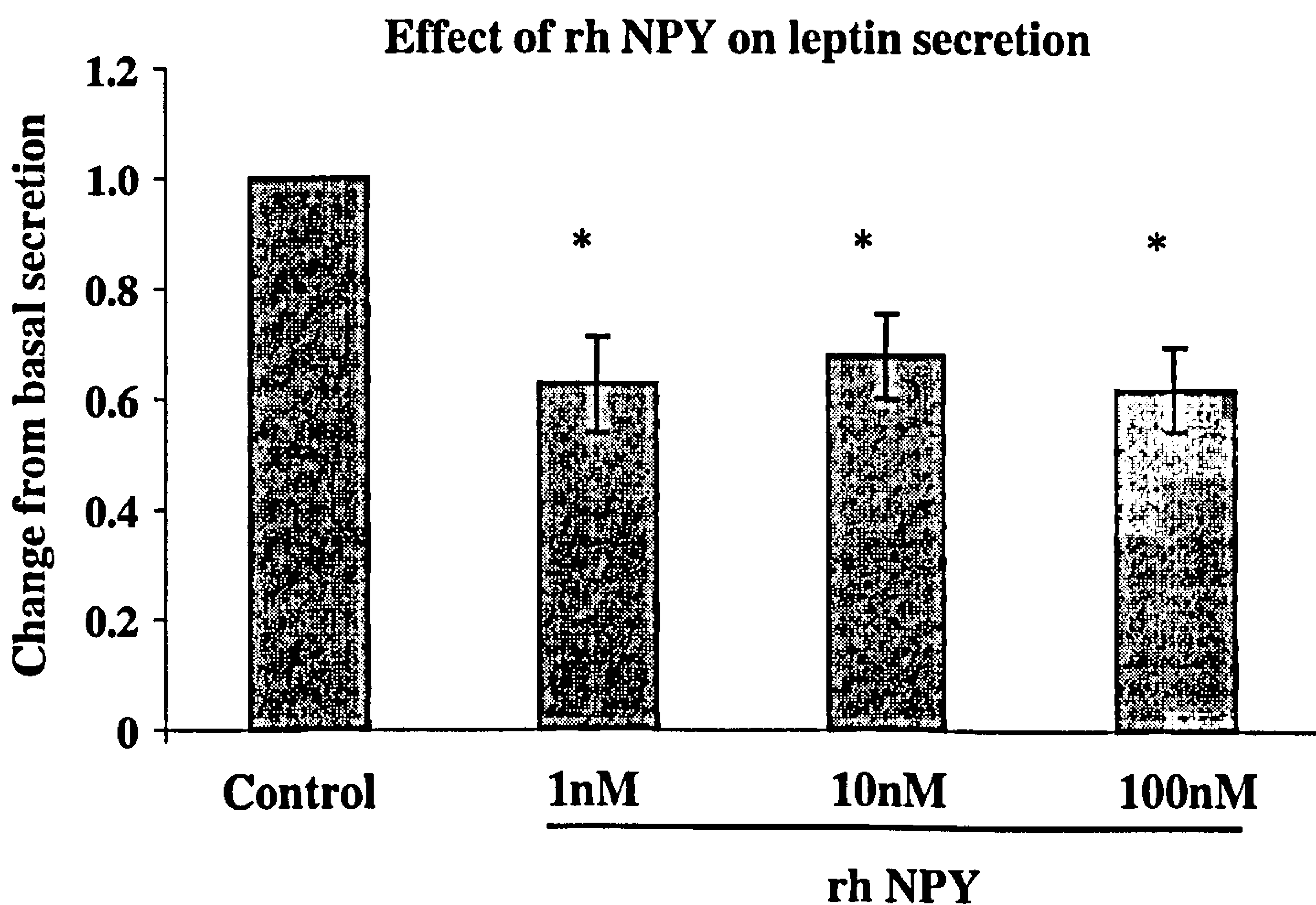


Figure 4.3.4.1.: Abd Sc adipocytes treated with rh NPY and the relative change of leptin secretion in comparison to basal secretion in the control media with adjusted value of 1, n=9, * $p<0.05$.

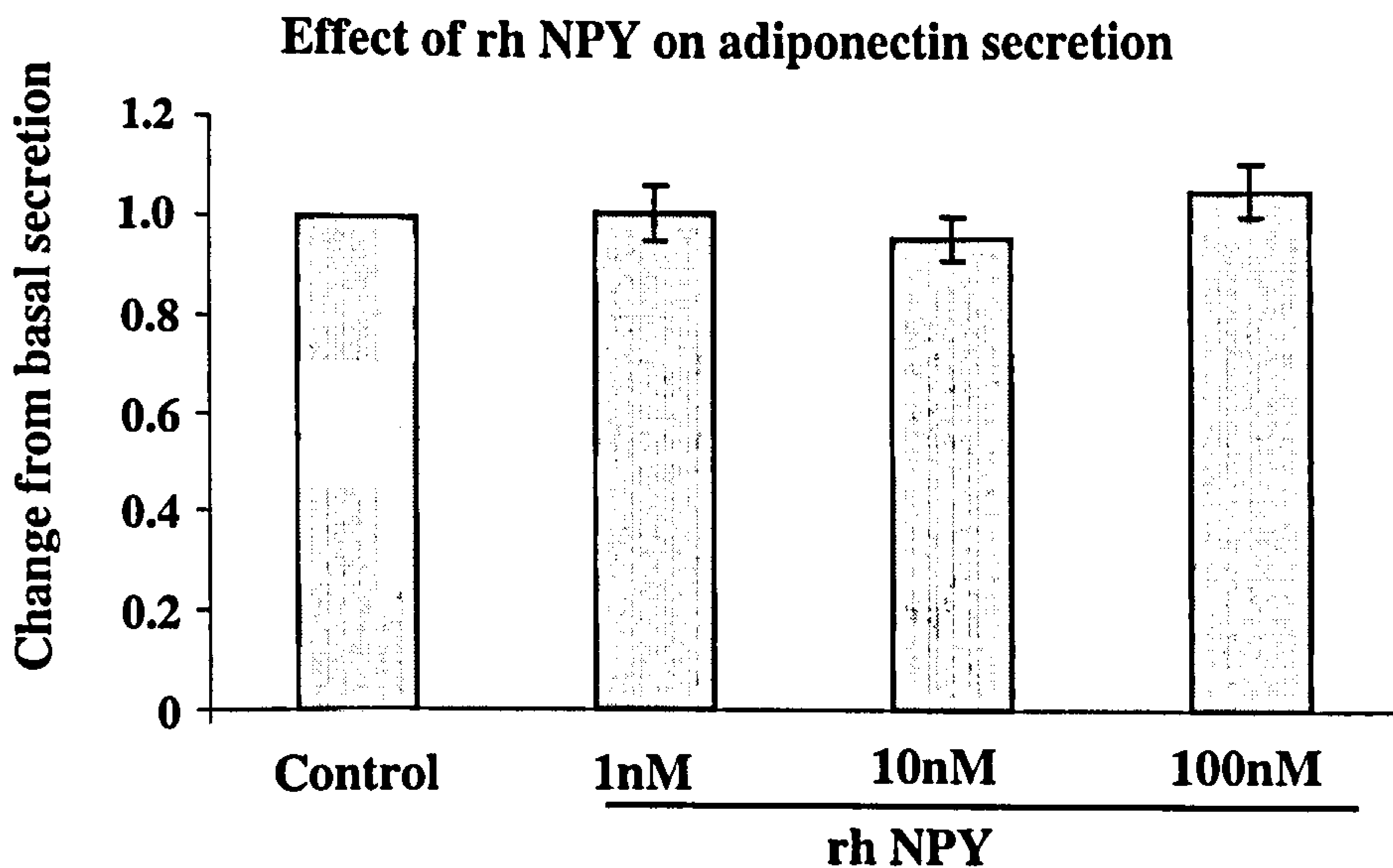


Figure 4.3.4.2.: Abd Sc adipocytes treated with rh NPY and the relative change of secretion of adiponectin in comparison to the basal secretion of control media with adjusted value of 1, n=9, p=NS.

In comparison, rh NPY treatment did not change basal adiponectin secretion (control 1; 1±0.06; 0.96±0.04; 1.08± 0.05 for NPY 1, 10 and 100nmol/L respectively, n=9, p=NS; Figure 4.3.4.2.).

4.3.5. Immunostaining of NPY

As demonstrated in Figure 4.3.5., NPY antibody (brown) is taken up in the cytoplasm of adipocytes. The staining of the rat brain with NPY, where this neurotransmitter is abundant, serves as a positive control.

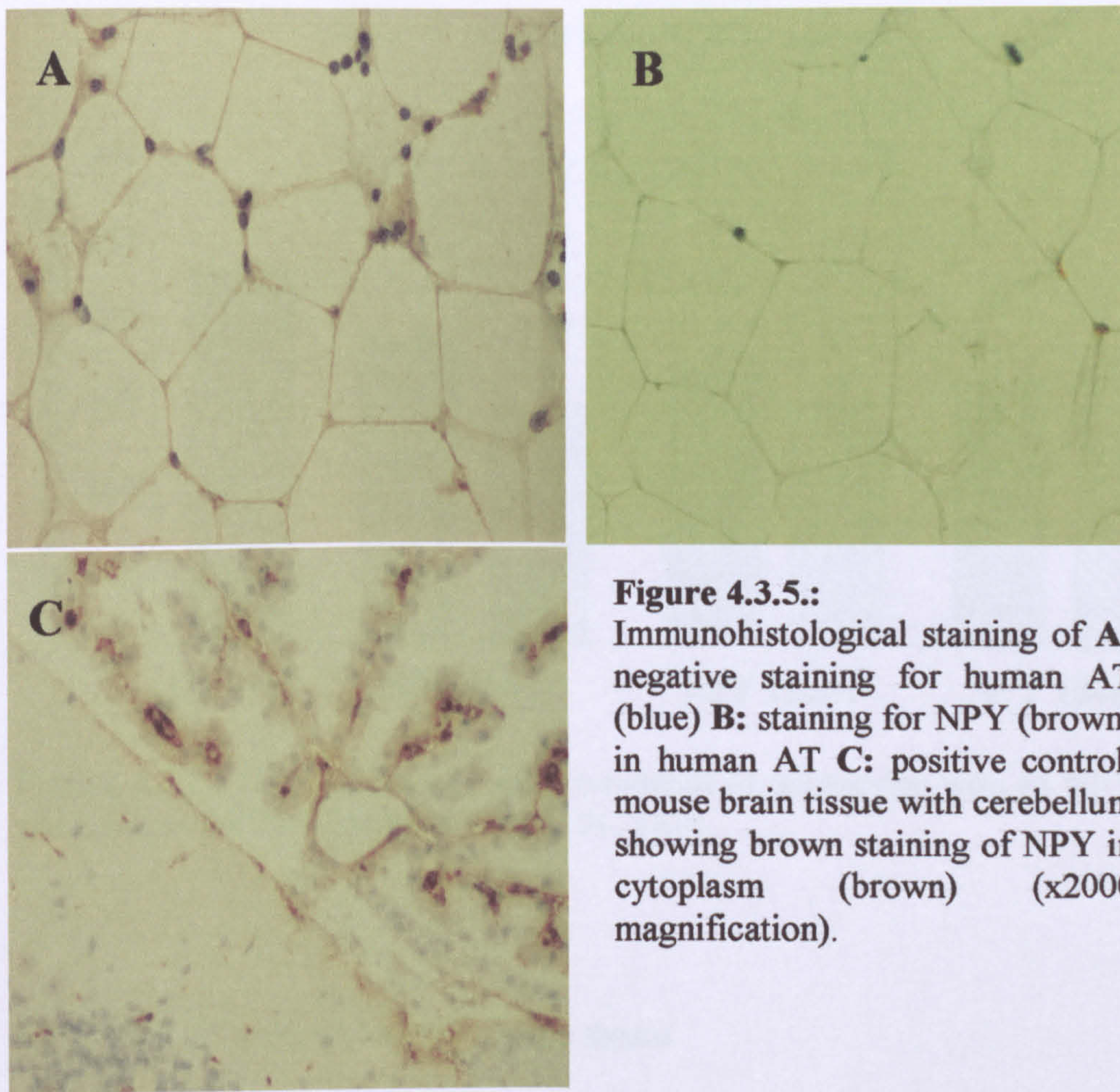


Figure 4.3.5.:
Immunohistological staining of **A:** negative staining for human AT (blue) **B:** staining for NPY (brown) in human AT **C:** positive control: mouse brain tissue with cerebellum showing brown staining of NPY in cytoplasm (brown) (x2000 magnification).

4.3.6. Glycerol secretion following NPY treatment

Abd Sc adipocytes treated with rh NPY were used to assess the antilipolytic action of NPY through glycerol release, which was significantly reduced with NPY (Control: 224±37µmol/L; NPY1nmol/L: 182±32µmol/L; NPY10nmol/L: 171.1±26µmol/L, NPY100nmol/L 161±27µmol/L, $p<0.01$, n=14). Addition of the DPP-IV inhibitor lead to a further decrease of glycerol secretion (NPY1nmol/L: 145±19µmol/L; NPY10nmol/L: 148.2±17µmol/L; NPY100nmol/L: 127±14µmol/L; $p<0.01$, n=14; Figure 4.3.6.).

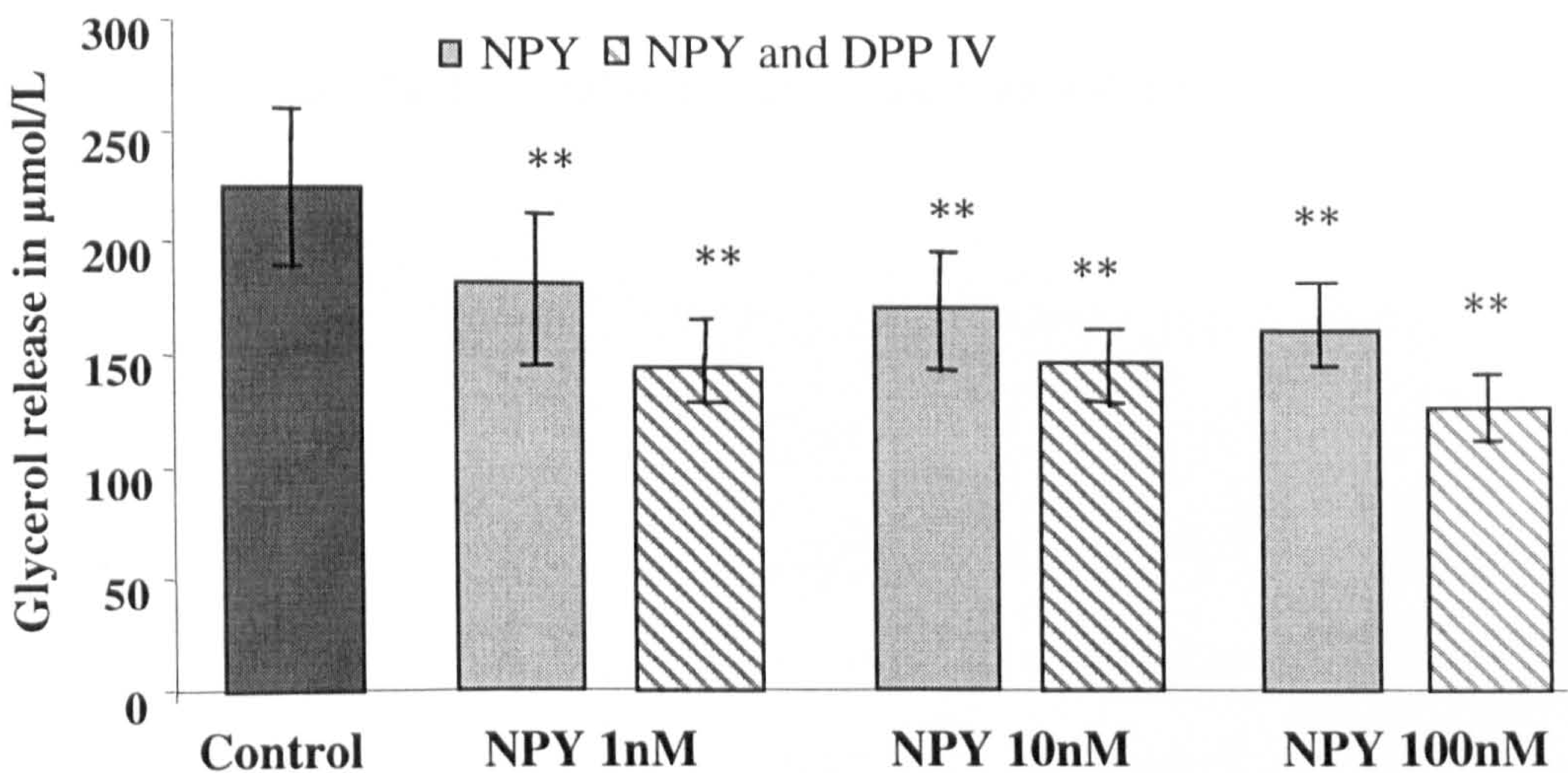


Figure 4.3.6.: Glycerol release after treatment of adipocytes with rh NPY with and without DPP-IV inhibitor, **p<0.01, n=14.

4.3.7. DPP-IV expression in adipose tissue

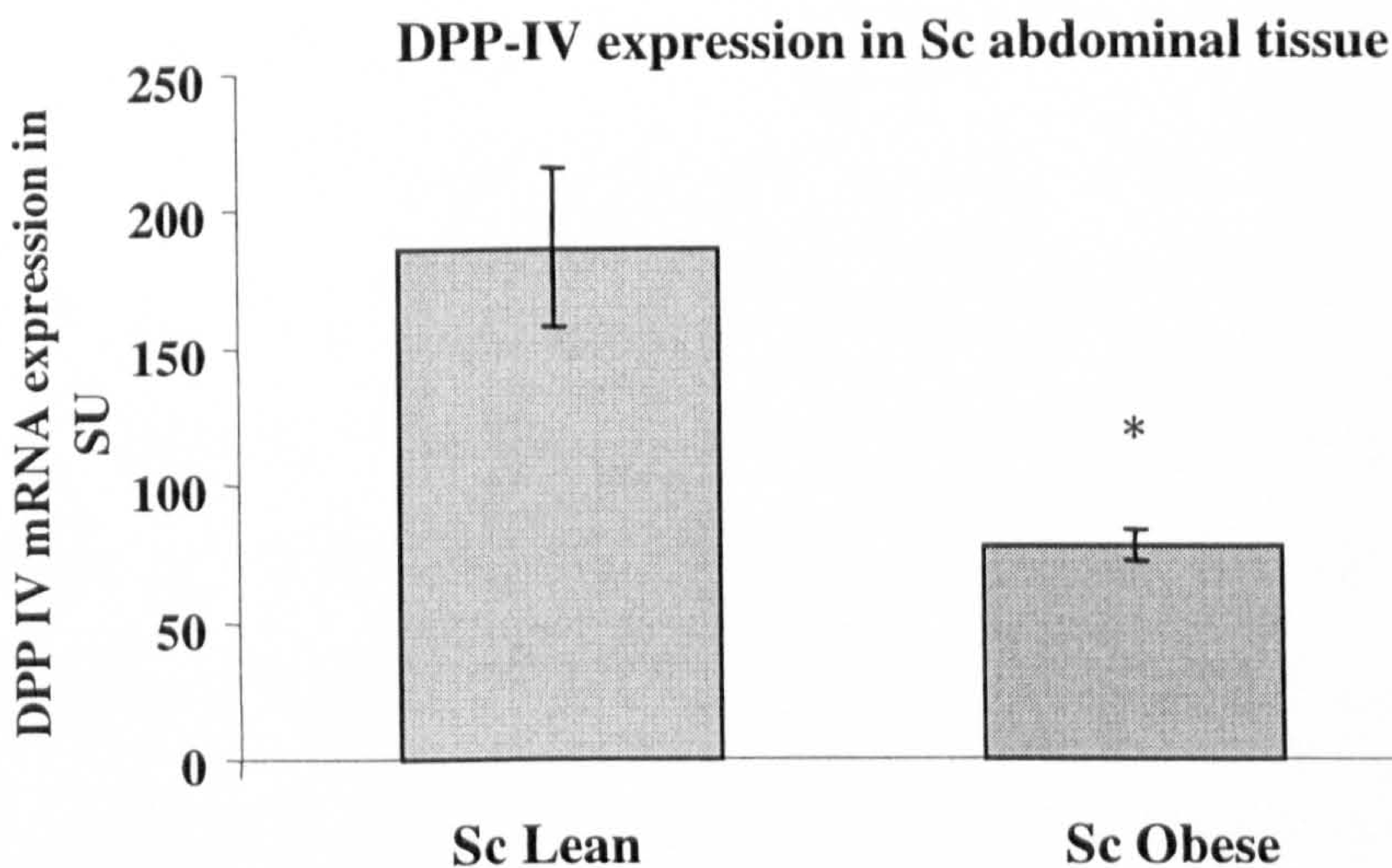


Figure 4.3.7.: Microarray expression of DPP-IV in lean versus obese Abd Sc AT, *p<0.05, n=10.

Microarray analyses revealed the expression of DPP-IV in abdominal, as well as omental AT. Whilst there was no significant difference between lean (BMI: 23.0±1.2 kg/m^2) and obese individuals (BM 30.3±3.1kg/m^2) in omental AT (not

shown), the relative expression of DPP-IV was much higher in the Abd Sc AT of lean individuals when compared to matched obese individuals (186±29 signal units (SU) versus 77±6SU, $p=0.018$, $n=10$) (see Figure 4.3.7).

4.4. Discussion

This is the first study to document protein expression of NPY in human AT and adipocytes. NPY was not only expressed within various adipose tissue depots, but also as a secreted protein in adipocyte conditioned media. Further evidence of the presence of NPY in human AT was documented through immunohistochemical staining of NPY in the cytoplasm of adipocytes. Other sources of NPY in cell culture are unlikely, as contamination of isolated adipocytes with blood or endothelial cells was previously excluded (Fisher, 2002). Contamination with neural cells is also unlikely and is supported by evidence that the SNS loses its capacity of NA release with coculture with other cell lines (Landis, 1990). However, analyses of the expression of MAP2, a marker of dendrites, synaptotagmin or synaptophysin, which are markers of neural innervation may further clarify which proportion of NPY in AT is derived from sympathetic innervation. The production of NPY by AT is further supported by a cDNA array study which found NPY in human visceral AT (Yang, 2003).

The findings of this study also show clear protein expression of prepro-NPY. Whilst the NPY ELISA has little cross reactivity with the prepro- hormone, the presence of converting enzymes for NPY, such as precursor convertase 2 (PC2) or PC3, which were previously found to be expressed in neuronal and endocrine cells of different species (Wulff, 1993) is likely, but remains to be proven.

WAT innervation was discussed in Chapter 1 and is in the majority by sympathetic nerves, but also sensory nerves mediated by CGRP and substance P (Shi, 2005). Turtzo (2001) compared the effect of coculture of sympathetic nerves with rodent 3T3L1 preadipocyte cell lines. Whilst the study differs with

use of rodent preadipocyte cell lines instead of human adipocytes, it is interesting to note that the NPY secretion in coculture was increased by up to 7.5 times in relation to pure NPY neuron secretion; the study further reported a small amount of NPY secretion by the adipocyte cell line itself. The distribution and function of NPY receptors differs amongst species. For instance, whilst work by Turtzo (2001) also found an antilipolytic effect of NPY based on glycerol secretion in the mouse adipocyte cell lines, Turtzo and its co-workers (2001) only found Y5 mRNA expression and no Y1, Y2 or Y4 mRNA expression in these mice. In comparison, the above experiments confirmed protein expression of Y1 and Y5 in human adipocytes, which is compatible with the finding of Y1 and Y5 mRNA expression identified by Serradeil-La Gal in human adipocytes (2000). Also, it is now believed, that Y1 may mediate the antilipolytic effect of NPY in rodents (Bradley, 2005) and humans (Serradeil-La Gal, 2000) as evident by elimination of NPY's antilipolytic effect with the use of Y1 receptor antagonists.

This study shows that inhibition of DPP-IV has significant effects on the NPY-mediated antilipolytic property, most likely mediated by preservation of the antilipolytic Y1 function of NPY. Furthermore, this study suggests that DPP-IV is differentially expressed in human AT depots and that AT is a potential site of DPP-IV secretion. DPP-IV gene expression appears to be downregulated in obesity, which could lead to augmentation of NPY's antilipolytic action. However, the expression of DPP-IV in the adipocyte needs further definition. These preliminary findings may imply that the therapeutic use of DPP-IV inhibitors may be ineffective in AT of obese subjects. In support of this, recent drug trials examining the effect of DPP-IV inhibitors failed to show a weight change in insulin resistant rats (Burkey, 2005) or T2DM subjects (Pi-Sunyer,

2007), who are usually overweight if not obese and as such our current data supplies a rationale for its lack of effectiveness.

This study further identified a depot-specific expression of NPY, with its highest expression in Abd Sc AT, which has been associated with a higher metabolic risk, in contrast to gluteo-femoral fat (Abate, 1996; Garg 2004) and which can contribute to more than 50% of total fat mass (Hattori, 1991). Whilst the sympathetic innervation was reported to be very high in visceral fat which has also highest lipolytic activity (Hoffstedt, 1997) which is further enhanced by increased ß-adrenoceptor sensitivity in obese male subjects (Hoffstedt, 1997), there may be separate regulation and secretion of adipocyte derived NPY, unrelated to the NPY release by SNS innervation.

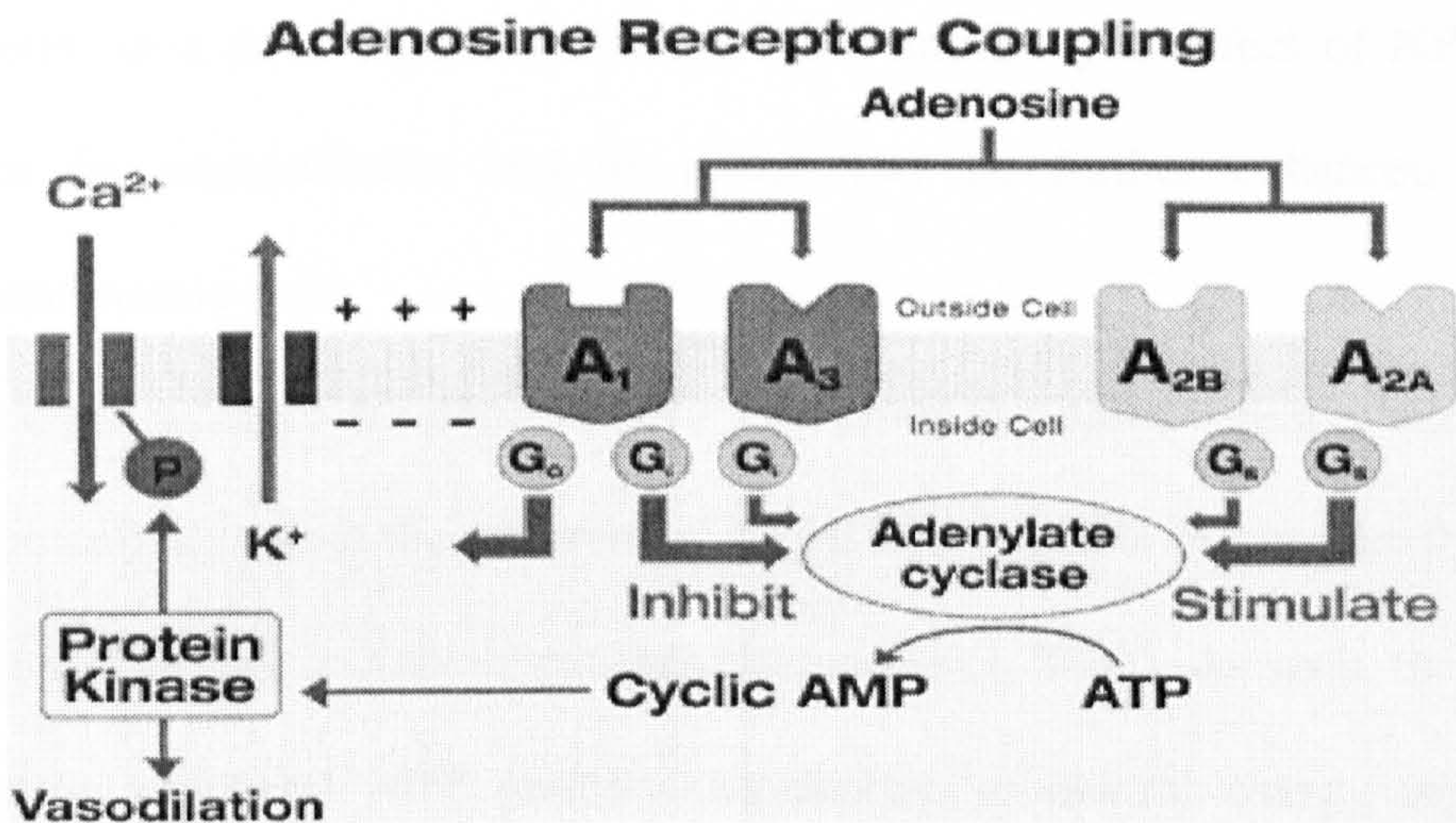


Figure 4.4.1.: Presynaptic adenosine receptors can differentially regulate the sympathetic co-transmission as shown in rodent vascular endothelium. Adenosine receptors A(2A) and A(3) inhibit the release of NA without affecting immunoreactive-NPY (Donoso, 2006).

However, even the SNS innervation may show differential regulation of the cotransmission of NE, a lipolytic neurotransmitter, and NPY which is anti-lipolytic by presynaptic adenosine receptors as demonstrated in vascular endothelium (Donoso, 2006). Some adenosine receptors can inhibit the release of NE, without affecting the immunoreactivity of NPY (see Figure 4.4.1). It

remains to be confirmed whether a similar regulation exists in human WAT. NPY serum levels are influenced by various cofounding factors, such as menstrual status, chronic stress, diabetes and hypertension (Baronowska, 2000). A review of the current literature fails to report a direct association with BMI for both NPY serum levels and cerebrospinal fluid (CSF) levels (Baranowska, 2000; Nam, 2001). However, elevated serum levels of NPY were reported in anorexic and morbidly obese subjects (Baranowska, 2000). Under consideration of known cofounding factors this study revealed no age or gender correlation, however, identified a positive correlation of NPY serum levels with BMI. Apart from leptin, which was previously shown to be upregulated by hyperinsulinaemia (Saladin, 1995), these studies also observed that NPY secretion was stimulated by insulin, in a dose dependent manner. The antilipolytic effect of NPY may enhance fat accumulation and its effect may be further enhanced in the hyperinsulinamic state.

It is unclear whether fat derived NPY can enter the blood stream; whilst this could potentially cause the observed elevated serum levels in chronically obese subjects and in type 2 diabetic patients (Baranowska, 2003). As such, the role of circulating peripheral NPY and its contribution to central energy regulation remains to be established. Circulating NPY is known to cross the blood brain barrier (Kastin, 1999) and AT derived NPY which could significantly raise circulating NPY in obesity may then elicit a central feedback mechanism on adipose tissue mass similar to leptin. The peripheral antilipolytic effect of insulin and its effect on NPY secretion are in contrast to the central insulin regulation, whereby insulin inhibits NPY gene expression (Gerozissis, 2004) and promotes weight loss. This may be explained by insulin promoting surplus energy storage

in adipocytes and fat accumulation with avoidance of further energy intake by reduction of appetite.

Thiazolidinediones (TZDs), such as RSG, are insulin-sensitisers with ß-cell preserving action (Ovalle, 2004). TZDs favourably increase circulating levels of adiponectin, which are amongst others cardioprotective (Tsuchida, 2005; Pischon, 2004). However, the use of TZDs is also associated with weight gain. This study did not find any effect of RSG on adipocyte derived-NPY secretion or adiponectin secretion, which further supports that RSG and NPY do not integrate a common pathway.

Finally, this study demonstrated a decrease of leptin secretion by NPY treatment. A downregulation of leptin appears necessary in order for NPY to have a significant effect on fat accumulation via its antilipolytic function (Wang, 2000). An upregulation of leptin, in turn, would not allow NPY to have a significant overall effect on the regulation of fat mass. Central action of leptin is known to promote peripheral sympathetic activity (Rayner, 2001); furthermore, coculture of adipocytes with sympathetic neurons was shown to decrease leptin release, as part of a potential feedback mechanism (Turtzo, 2001). This is in contradiction with previous studies, which have established an upregulation of leptin by NPY in cultured adipocytes (Dyer, 1997; Serradeil-Le Gal, 2000). However, the experimental conditions differ greatly following use of either sheep adipocytes after *iv* administration of NPY, or use of preadipocyte cell lines (Serradeil-Le Gal, 2000). Whilst it is difficult to determine the complex interplay of these factors on adipogenesis, a possible resistance to certain factors, *i.e.*, leptin, may

be present in isolated adipocytes of obese subjects and complicate the interpretation.

In conclusion, NPY protein is expressed in AT and adipocytes in a depot specific manner, suggesting that NPY is not only a neurotransmitter but is also actively produced by adipocytes themselves. NPY may have a role in insulin resistance. Insulin upregulates NPY adipocyte secretion and NPY reduces leptin secretion. Additionally, serum NPY levels appear to be positively correlated with BMI. NPY's antilipolytic effect may aggravate fat mass accumulation and may further, in turn, worsen insulin resistance. Whether the contribution of AT-derived NPY in the general circulation elicits central feedback remains to be determined. This study suggests that AT may have a similar NPY regulation to endothelium (Zukowska-Grojec, 1998b), in that it possesses an organ specific autocrine system for NPY, which includes site-specific receptors, the NPY cleaving protease DPP-IV and its own production of NPY.

Chapter 5

Ghrelin in adipose tissue

5.1. Introduction

Ghrelin is a 28 amino-acid-peptide and ligand for the orphan growth hormone secretagogue receptor GHSR (Kojima, 1999). Whilst its best characterised effect is the increase in appetite via hypothalamic action (Tschop, 2000), ghrelin is predominantly derived from the stomach and gut (Kojima, 1999) and can further mediate various peripheral effects (Kojima, 2005). Human studies to date have demonstrated that *iv* administration of ghrelin stimulates appetite and food intake (Wren, 2001); additionally, circulating ghrelin levels are at their highest prior to food intake and drop subsequently, compatible with their roles as 'meal initiators' (Cummings, 2001). The orexigenic effect is thought to be mediated by the action of octanylated ghrelin in the arcuate nucleus (ARC) of the hypothalamus (see Chapter 1). Octanylated ghrelin serves as central neurotransmitter; which is complicated by the observation that ghrelin may also be locally/centrally synthesised (Kojima, 1999; Korbonits, 2001), though this central production is thought to contribute little to its systemic concentration (van der Toorn, 2002). Des-acetylated ghrelin (also known as des-octanylated ghrelin) is the major circulating form of ghrelin (Ghigo, 2005). With the absence of the octanoyl-modification of the third serine residue, des-acetylated ghrelin is unable to activate GHS-R1a and mediate endocrine action (*e.g.*, is unable to stimulate the release of GH, prolactin and ACTH) (Broglio, 2004). Therefore des-acetylated ghrelin was initially considered to lack not only any endocrine effect but to be biologically inactive (Hosoda, 2000). More recent evidence, however, suggests that both octanylated and des-acetylated ghrelin may mediate peripheral biological actions (Thompson, 2004); furthermore, there is a suggestion that both can act antagonistically (Broglio, 2004).

However, little is known about the exact role of the two distinct isoforms or the enzyme responsible for the posttranslational modification of ghrelin (van der Lely, 2004).

Furthermore, few studies have established how this orexigenic neurotransmitter exerts feedback on peripheral energy regulation, in particular, on adipose tissue storage in humans. It has been demonstrated in rats that *icv* injection of ghrelin affects AT, and most likely establishes crosstalk with AT, mediated by sympathetic innervation similar to leptin (Theander-Carrillo, 2002). Rodent studies also suggest that ghrelin elicits an antilipolytic effect (Morimoto, 1998) and promotes adipogenesis in the bone marrow (Thompson, 2004); these effects are mediated by both octanylated and des-acetylated ghrelin in rodents (Muccioli, 2004; Thompson, 2004). GHS-R antagonists have been shown to reduce fat mass *in vitro* (Asakawa, 2003); however, the use of potent GHS-R agonists does not enhance the adipogenic action of ghrelin in rodents (Thompson, 2004). The GHS-R has not been identified in human AT (Gnanapavan, 2002) and thus the actual receptor through which ghrelin and more so, the des-octanylated ghrelin isoforms, could mediate an effect on fat metabolism is unrelsoved. Recent studies suggest that ghrelin can also act as central ligand of the Y1 receptor (Toshinai, 2003; Tebbe, 2005). However, there are no studies which have determined whether ghrelin is able to mediate peripheral effects through Y1. The receptor Y1 was discussed in more detail in Chapter 4 as a mediator of NPY's action on peripheral fat metabolism. Y1 could as such potentially mediate action of ghrelin in fat similar to NPY and control one of the most important two enzymes coordinating lipolysis and

lipogenesis which are the hormone sensitive lipase (HSL) and lipoprotein lipase (LPL) respectively (Sztalryd, 1995).

Therefore, the aims of this chapter are to elucidate the expression and distribution of ghrelin in human AT and adipocytes and the role of increasing adiposity on the level of ghrelin expression. Furthermore, this study will examine whether octanylated and des-acetylated ghrelin differ in their role in human fat metabolism and whether Y1 could be mediating ghrelins' antilipolytic action.

5.2. Methods

5.2.1. Subjects

AT samples were obtained from twenty consenting white, female subjects undergoing elective surgery. Mean age: 46±6.8years (mean±SD), BMI:25.6±4.96kg/m^2. Abdominal subcutaneous (AbdSc), omental (Om) and thigh tissue (Th) samples were collected with the approval of the South Birmingham Ethics Committee. Subjects on endocrine therapy (e.g., steroids, HRT, thyroxine) and patients with malignant diseases were excluded.

Fasting serum samples were taken from 26 healthy subjects with unchanged body weight in the previous 6 months constituting of 13 men (age:38.1±12.3years, BMI: 26.0±4.0kg/m^2) and 13 women (age: 37±8.9years, BMI: 24.6±4.4kg/m^2). Blood samples were centrifuged at 700 x g for 20 min and serum stored at -80°C till used for further analysis.

5.2.2. Adipocyte isolation and cell treatment

The isolation of adipocytes and their subsequent cell-culture was followed by similar principles as described in Chapter 4. Freshly prepared abdominal subcutaneous adipocytes suspended in cell culture medium were treated with human recombinant (rh) octanylated ghrelin (Diagnostic Systems Laboratories, Inc, USA; 1,10 and 100 nM) or rh des-acetylated ghrelin (1,10 and 100nM) for 48 hr. These ghrelin treatments were run with and without addition of the NPY receptor Y1 blocker (BIBP3226, Sanofi-Synethe-labo, France) at a dose of 100nM for full blockage as derived from receptor binding studies (Entzeroth, 1995). Untreated adipocytes were used as controls.

Following incubation of adipocytes (37°C/5%CO_2), conditioned media and adipocytes were separated by centrifugation (360 x g for 2 min). Media was then removed, separated into aliquots, and then stored at –80°C. The remaining adipocytes were treated with RIPA buffer (see Chapter 2) and frozen to be later used for Western blot analyses.

5.2.3. Protein extraction

Part of the adipose tissue was flash frozen and then stored at –70°C. Prior to use for Western blot analysis, a small piece of frozen adipose tissue was homogenised and extracted with RIPA buffer, containing: 1x PBS, 1% NonIdet P40, 0.5% Sodium Deoxycholate, 0.1% SDS, 1 complete mini protease cocktail (Roche Molecular Biochemicals, Germany) and 100 μg/mL ALLN (Calbiochem, San Diego, USA). Protein was then quantified using a Bio-Rad DC protein assay kit (BioRad, Hercules, California, USA).

5.2.4. Western blot analysis of ghrelin

Homogenised human adipose tissue (5g) and isolated Abd Sc adipocytes (200μL) were extracted in 400μL of RIPA buffer. Adipose tissue protein and adipocyte protein were quantified and loaded on a gel and separated by SDS-PAGE using a 15% gel. All samples were heated for 5 min at 95°C in a non-reducing loading buffer. Pre-stained molecular weight markers (BioRad) were used as standards. Proteins were transferred from the polyacrylamide gels to PVDF membranes by electroblotting in a vertical transfer tank at 140V for 1.30 hr. Membranes were blocked overnight at 4°C in phosphate buffered saline containing Tween 20 (PBS-T: PBS + 0.05% Tween 20; Sigma) containing 10%

(w/v) non-fat milk powder (Marvel, Moreton, Merseyside, UK). Membranes were then treated with primary antibody: mouse monoclonal Ghrelin antibody (Abcam, Inc.) (1:1000); then developed with a conjugated mouse HRP secondary antibody in concentrations of 1:80,000 diluted in PBS with 0.5%Tween. The ghrelin precursor protein (12kDa) was detected by chemiluminescent assay ECL^+ (Amersham, Little Chalfont, UK), which enabled visualisation following exposure to X-ray film and the band intensity was determined by densitometry. Equal loading was ensured with use of the α-tubulin (50kDa) antibody (Abcam, Inc), which required a repeat Western blot after stripping the membrane of the initial ghrelin primary and secondary antibody as described in Chapter 2.

5.2.5. Glycerol assay:

Glycerol is released as a by-product of lipolysis. After the above described 48 hr incubation with treatments, the infranatant containing media was used to assess the glycerol content using a commercially available colorimetric kit (Randox Laboratories, UK). The sensitivity of the assay, according to the manufacturer's protocol was estimated to be between 0-100mmol.

5.2.6. Western blotting for HSL and LPL

Cell treatments of isolated mature abdominal subcutaneous adipocytes with varying doses of octanylated and des-acetylated ghrelin were compared to control for A) hormone sensitive lipase (HSL 84kDa) and B) lipoprotein lipase (LPL 56kDa). Western blot analysis, similar to above, was performed, with loading of 20µg of protein and buffer containing dithiothreitol (DTT)

100nmol/L (Sigma, UK) and separated using 10% SDS-PAGE gels at 140V for 1.5 hr. After electrophoresis and transfer, as described above, membranes were blocked overnight at 4°C with continual motion. For LPL detection, membranes were incubated with LPL antibody (R&D systems, USA) and diluted 1:500 in PBS-T. The primary antibody was then detected by application of a secondary antibody (anti-sheep/goat IgG) conjugated to horseradish peroxidase (HRP) (The Binding Site, Birmingham, UK) diluted 1:2,500 in PBS-T.

The method was similar for HSL which was produced using a standard procedure previously detailed (Botion, 1999). The dilution for the primary antibody was 1:5000 in PBS-T and the secondary antibody (anti-rabbit IgG) conjugated to horseradish peroxidase (HRP) (The Binding Site, Birmingham, UK) was diluted 1:60,000 in PBS-T. Membranes were developed with chemiluminescent assay ECL/ECL$^+$ (Amersham, Little Chalfont, UK) and the bands measured with densitometry. All experiments were carried out in triplicate.

5.2.7. Serum ghrelin analysis and assay validation

The serum content of total ghrelin was assayed using the commercially available total ghrelin ELISA kit (Diagnostic Systems Laboratories, Inc., USA), sensitivity: 6.2-1800 pg/mL, intra-assay variation 2-10% and inter-assay variation 5-15%. Rh des-acetylated and octanylated ghrelin (Diagnostic Systems Laboratories, Inc, USA) were used for recovery study in the assessment of this ELISA kit. Concentrations of 10, 50 and 100pg/mL of des-acetylated and octanylated ghrelin were added to pooled serum (26.8pg/mL) and the observed values correlated with expected values. The recovery for

10pg/mL des-acetylated ghrelin was 95-99% efficient (for recovery of 10pg/mL we expected: 36.8 pg/mL and observed: 35.6±0.56pg/mL). For 10 pg/mL octanylated ghrelin the recovery was: 91-99% (expected: 36.8g/mL; observed: 34.6±1.15pg/mL).

5.2.8. Statistical analysis

Autoradiographs were quantified by densitometry. For assessment of protein expression between depots and lean and obese subgroups, statistical analysis was undertaken with a t-test. ANOVA was used for comparison of cell treatments with control for each group compared to each treatment set. Serum levels and BMI correlations were determined with a Pearson correlation. P values of less than 0.05 were considered as statistically significant. Values are expressed as mean +/- standard error (SEM) unless otherwise stated.

5.3. Results

5.3.1. Ghrelin protein expression and depot difference

Protein studies determined that prepro-Ghrelin (12kDa) and mature Ghrelin (4kDa) were expressed in adipose tissue and isolated adipocytes (Figure 5.3.1.1.). Western blot analyses further revealed depot-specific differences of ghrelin expression with highest expression in Abd Sc adipose tissue in comparison to omental (Om) AT and thigh AT (Abd Sc: 3.05±0.55OD (mean±SEM), Om: 1.5± 0.5OD, thigh: 1.0±0.4OD, $p<0.05$, $n=8$) (Figure 5.3.1.2.).

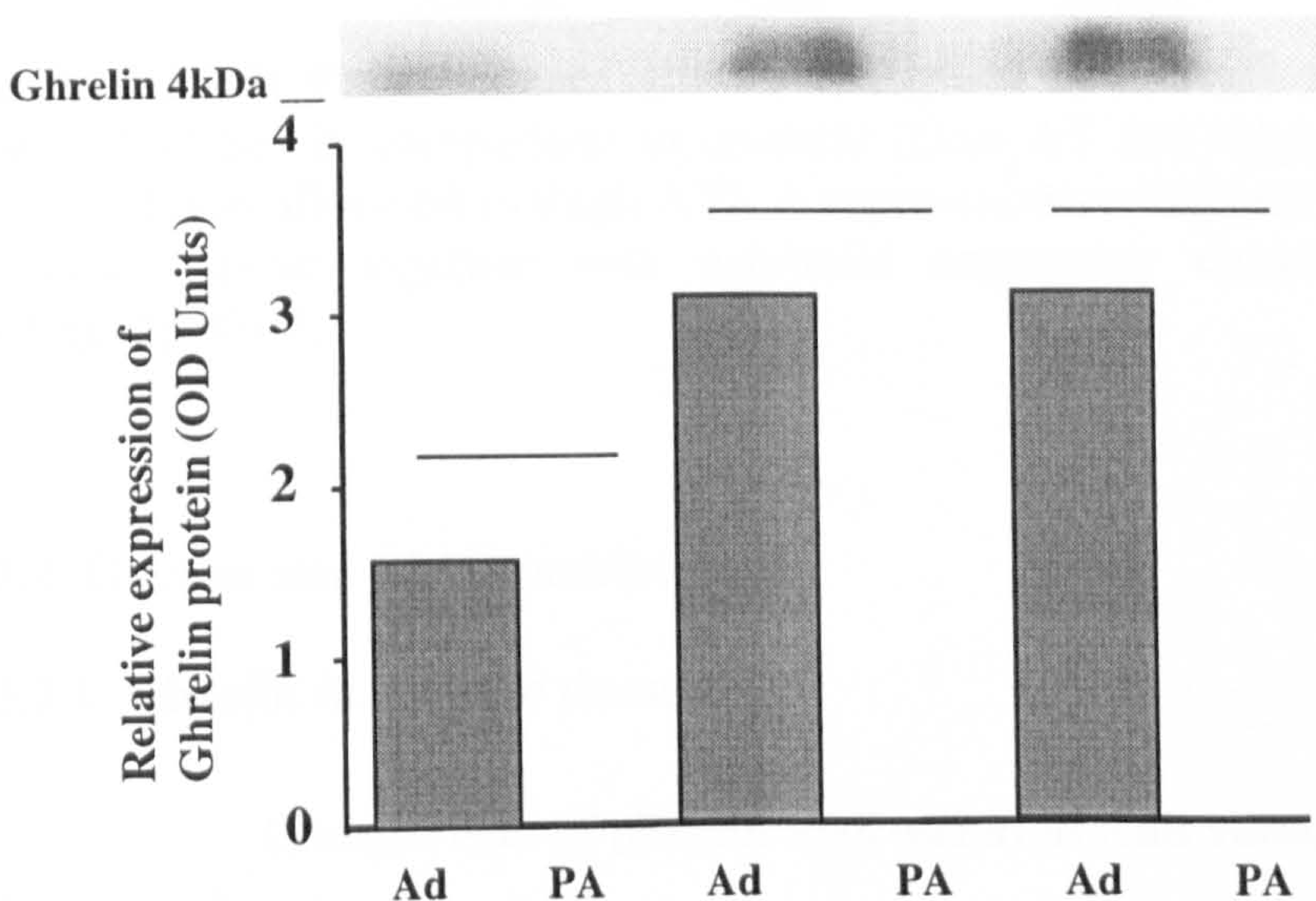


Figure 5.3.1.1: Protein expression of ghrelin in human abdominal subcutaneous adipocytes (Ad), but not pre-adipocytes (PA), as shown with three paired samples.

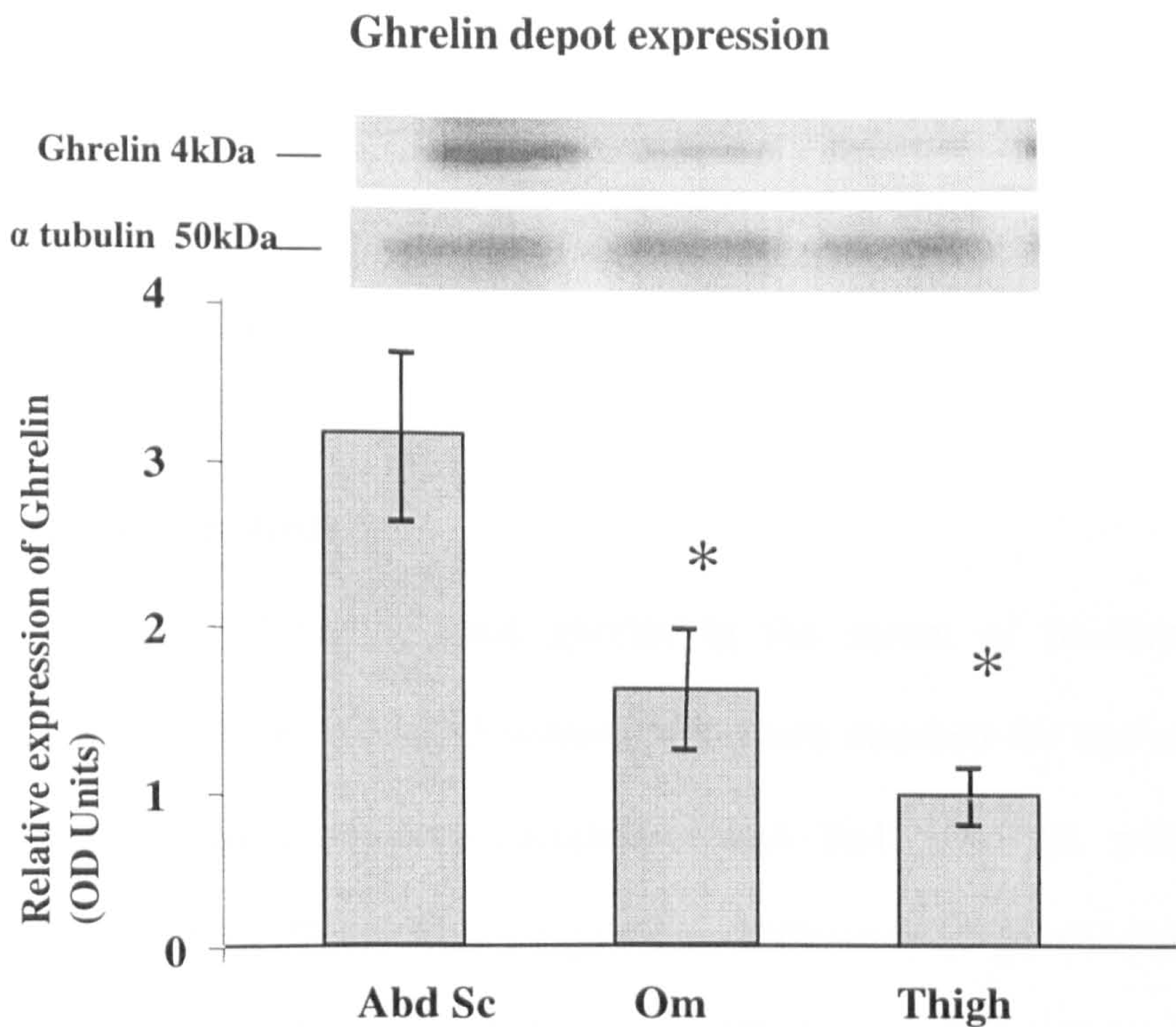


Figure 5.3.1.1: Expression of ghrelin is highest in abdominal subcutaneous tissue (Abd Sc) in comparison to omental (Om) AT and thigh AT (Arbitrary value of 1 was allocated to thigh AT). A representative Western blot for ghrelin is shown above together with α-tubulin expression demonstrating equal loading, *p<0.05.

5.3.2. Ghrelin and BMI correlation

5.3.2.1. Ghrelin in adipose tissue:

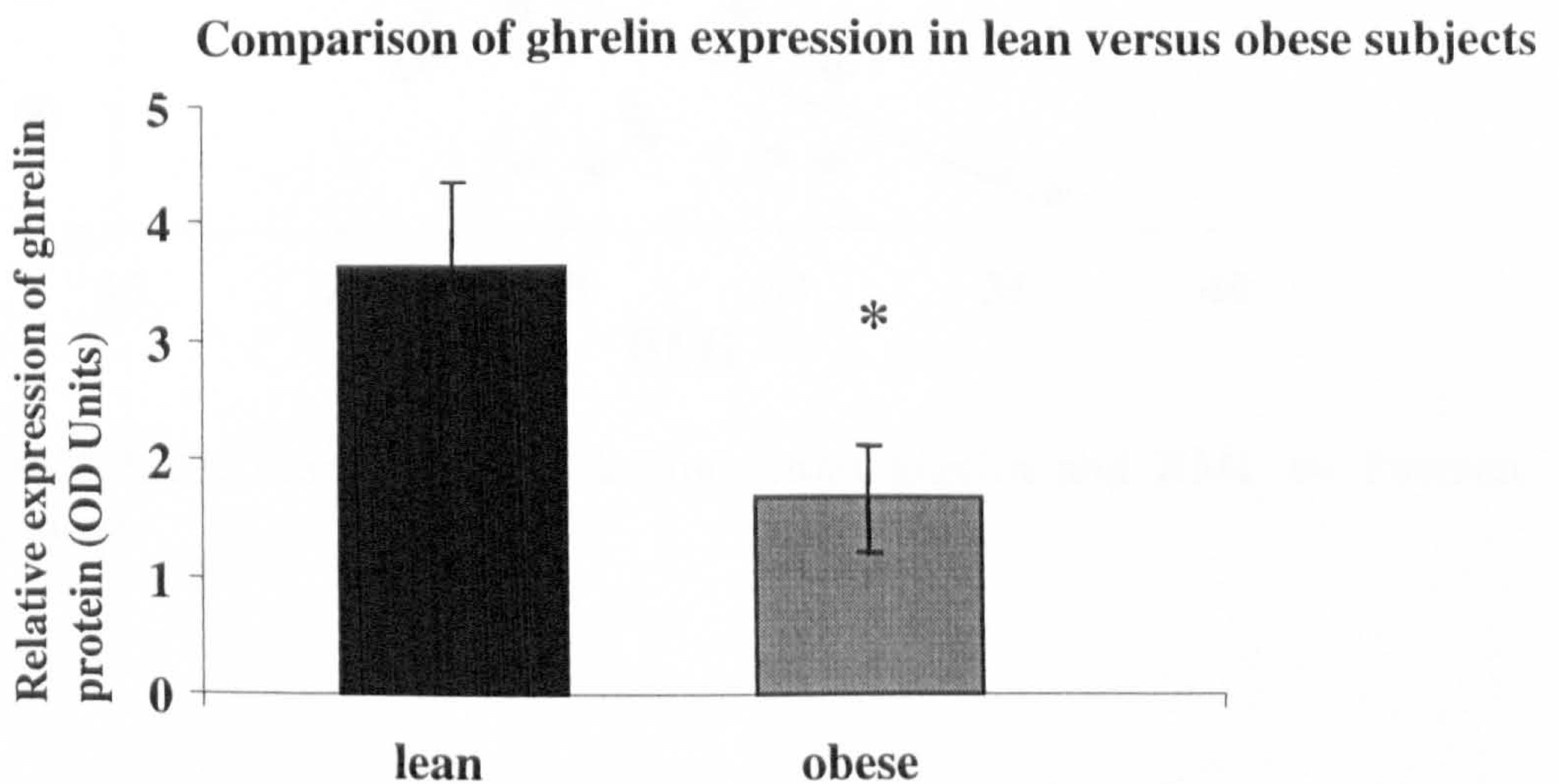


Figure 5.3.2.1.: Relative expression of ghrelin in adipose tissue of lean versus obese women *p<0.05, n=6 paired subjects.

Comparison of ghrelin expression between 6 lean (mean BMI 22.4kg/m^2) and 6 overweight/obese women (mean BMI 30.9kg/m^2) showed a significant negative correlation (lean: 3.6±0.74OD and obese: 1.64±0.45OD respectively; $p<0.05$) (Figure 5.3.2.1.).

5.3.2.2. Serum Ghrelin

The analysis of fasting total ghrelin in the serum of healthy individuals consisting of 13 men and 13 women who were matched for age and BMI (see above) showed a negative correlation with BMI ($r=-0.54$, $p<0.001$, $n=26$) (Figure 5.3.2.2.). There was a significant difference of ghrelin levels between gender (women: 91.7±16pg/mL (mean±SEM), men: 54.8±7.2pg/ml, $p<0.05$) but this was not correlated with age ($r=-0.1$, p=NS).

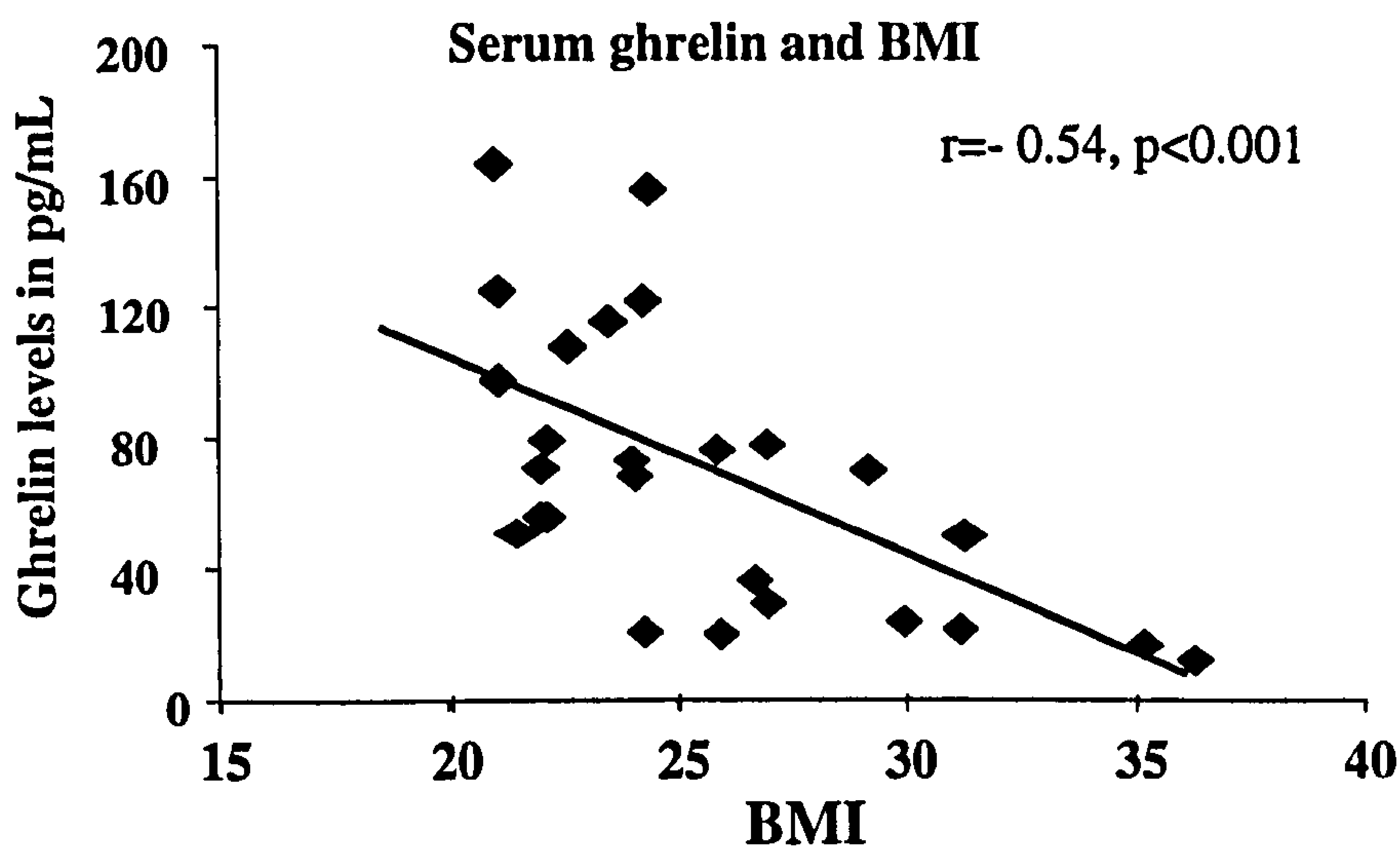


Figure 5.3.2.2: Correlation of fasting serum ghrelin and BMI. r= Pearson correlation

5.3.3. Ghrelin and lipid metabolism

The influence of the acetylation status of ghrelin on lipolysis and lipogenesis in cultured human adipocytes was distinguished by separate analysis of its effect on HSL, LPL and glycerol release.

5.3.3.1. Lipolysis

5.3.3.1.1. Glycerol release

Isolated Abd Sc adipocytes were treated with increasing doses of ghrelin and compared for glycerol release as a by-product of lipolysis. There was no significant down regulation of glycerol release for octanylated ghrelin (Control: 286±58μl/L; O-Ghrelin 1nM: 207±31μl/L; O-Ghrelin 10nM: 241.4±46μl/L; O-Ghrelin 100nM: 209±33μl/L, p=NS, n=7). However, des-acetylated ghrelin significantly suppressed glycerol release (Control: 286±58μl/L; Des-Ghrelin 1nM: 224±38μl/L; Des-Ghrelin 10nM: 229±23μl/L; Des-Ghrelin 100nM: 172±13μl/L, $p<0.05$, n=7).

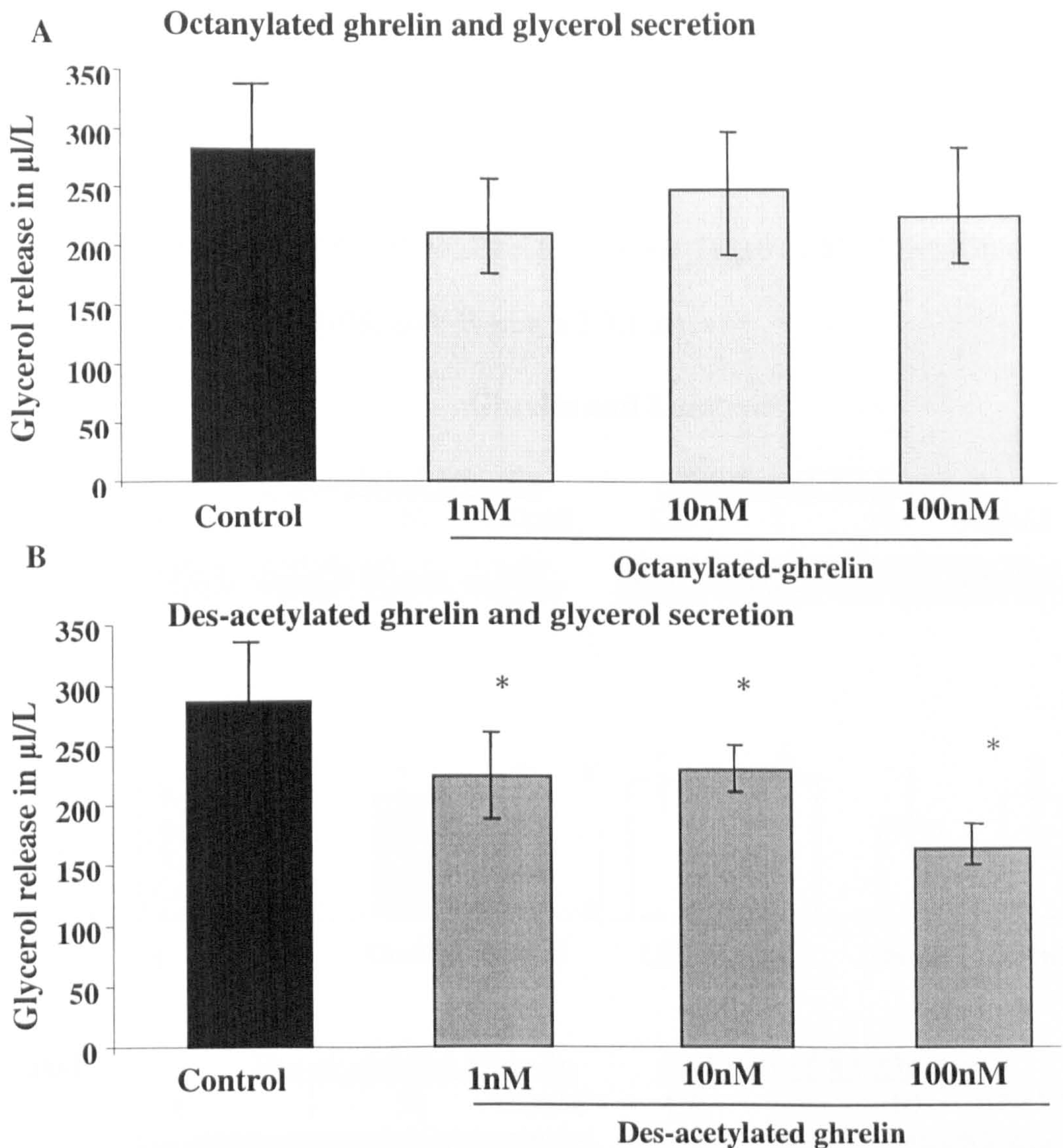


Figure 5.3.3.1.1: Glycerol release after adipocyte cell treatment with increasing doses of **A:** Octanylated and **B**: Des-acetylated ghrelin. Statistical analysis was performed using ANOVA,*p<0.05, n=7.

5.3.3.1.2. HSL production

There was no significant down regulation of HSL expression by octanylated ghrelin (Control: 1±0.3; O-Ghrelin 1nM: 1.1±0.4OD; O-Ghrelin 10nM: 0.9±0.3OD; O-Ghrelin 100nM: 0.8±0.4OD, p=NS, n=4). Addition of the Y1 blocker BIBP3226 did not significantly reduce HSL expression (CB: 1±0.2OD; BO-Ghrelin 1nM: 1±0.3OD; BO-Ghrelin 10nM: 0.7±0.35OD; BO-Ghrelin 100nM: 0.9±0.3OD, p=NS, n=4). However, des-acetylated ghrelin significantly

suppressed HSL expression (C: 1±0.3OD; Des-Ghrelin 1nM: 0.8±0.3OD; Des-Ghrelin 10nM: 0.6±0.1OD; Des-Ghrelin 100nM: 0.6±0.1OD, p<0.05, n=4). Addition of Y1-Blocker did not change this pattern (CB:1±0.3OD; BDes-Ghrelin 1nM: 1.1±0.2OD; BDes-Ghrelin 10nM: 0.8±0.1OD; BDes-Ghrelin 100nM: 0.6±0.1OD, p<0.05, n=4; Figure 5.3.3.1.2.).

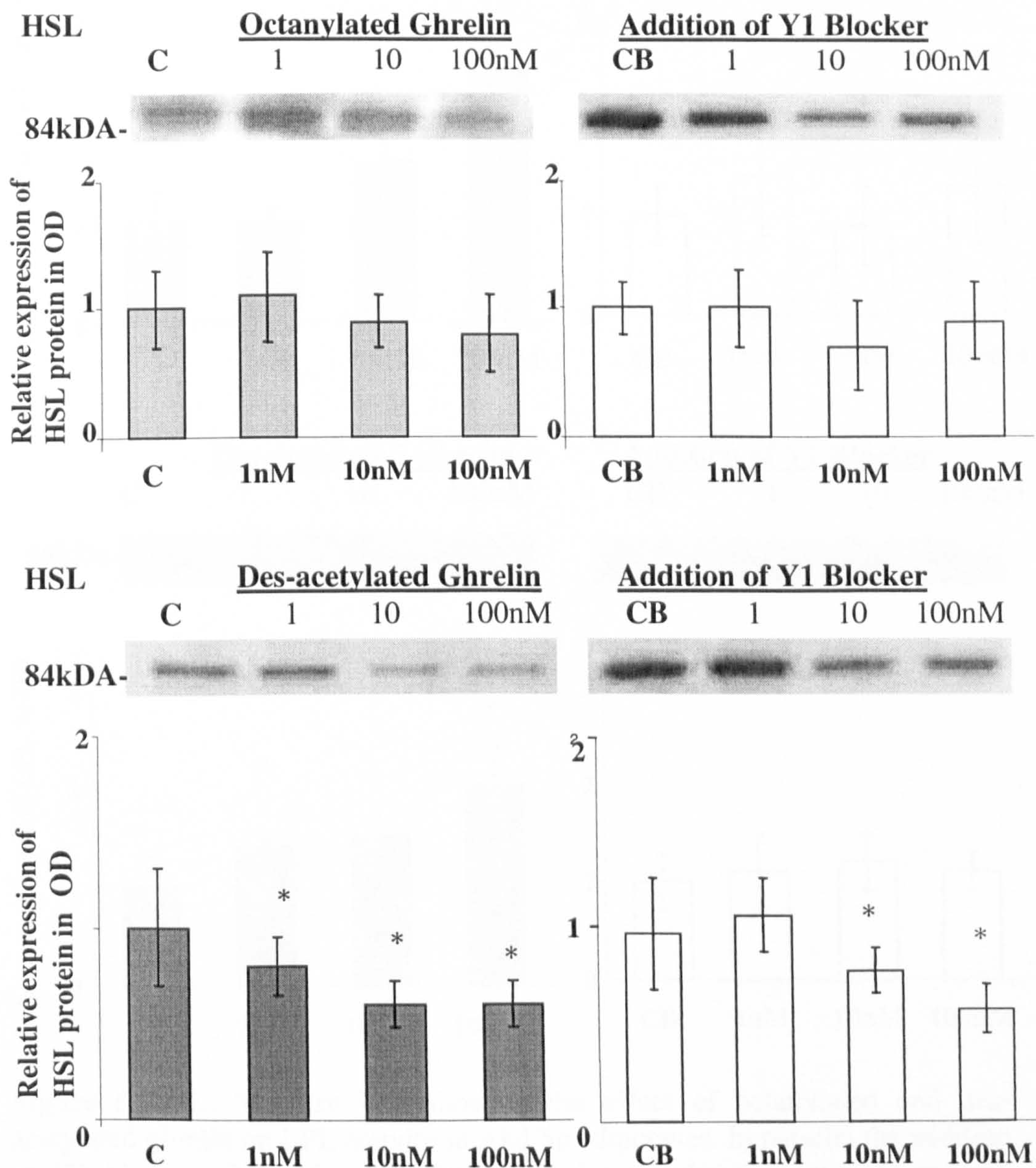


Figure 5.3.3.1.2.: Western blot showing the effect of octanylated and des-acetylated ghrelin on HSL activity. On the right side of the diagram the addition of Y1 blocker is demonstrated accordingly, n=4. C=control, CB=control with addition of Y1 blocker. The controls were given the arbitrary value of 1, n=4.*p<0.05

5.3.3.2. Lipogenesis

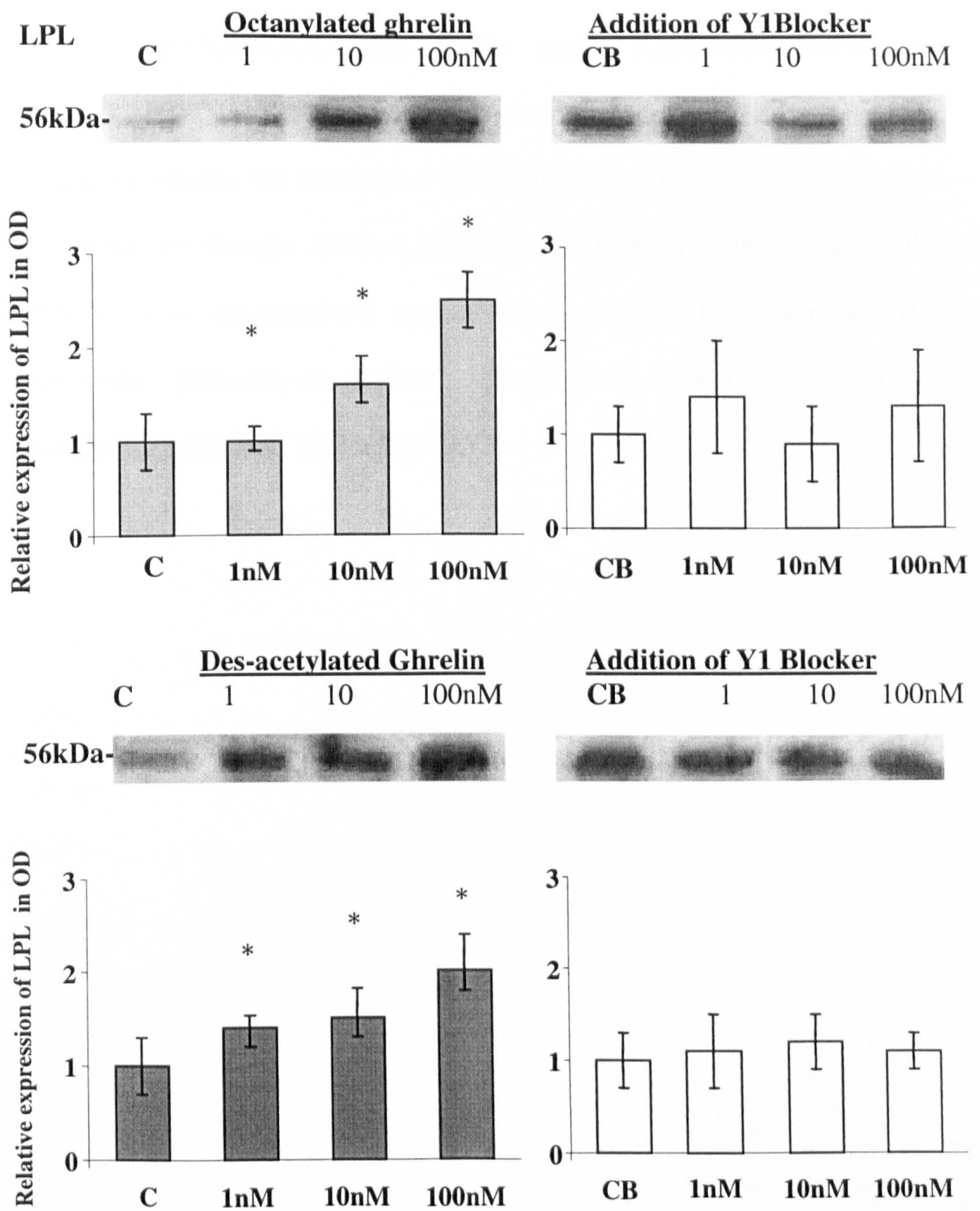


Figure 5.3.3.2.: Western blot showing the effect of octanylated and des-acetylated ghrelin on LPL activity in Abd Sc adipocytes. In parallel the addition of Y1 blocker alongside octanylated and des-acetylated ghrelin. C=control, CB=control with addition of Y1 blocker. The controls were given the arbitrary value of 1, n=4,*p<0.05.

Both octanylated and des-acetylated ghrelin show a significant increase of LPL production (C: 1±0.3; O-Ghrelin 1nM: 1.0±0.4OD; O-Ghrelin 10nM: 1.6±0.5OD; O-Ghrelin 100nM: 2.5±0.3OD, $p<0.05$, n=4; C: 1±0.3OD; Des-Ghrelin 1nM: 1.4±0.2OD; Des-Ghrelin 10nM: 1.5±0.4OD; Des-Ghrelin 100nM: 0.2±0.4OD, $p<0.05$, n=4)). This effect was not present by use of the Y1 receptor blocker for octanylated ghrelin (CB: 1±0.3OD; BO-Ghrelin 1nM: 1.4±0.6OD; BO-Ghrelin 10nM: 0.9±0.4OD; BO-Ghrelin 100nM: 1.3±0.6OD, p=NS, n=4) or des-acetylated ghrelin (CB: 1±0.3OD; BDes-Ghrelin 1nM: 1.1±0.4OD; BDes-Ghrelin 10nM: 1.2±0.3OD; BDes-Ghrelin 100nM: 1.1±0.2OD, p=NS, n=4; Figure 5.3.3.2.).

5.4. Discussion

This is the first study to show the protein expression of the neurotransmitter ghrelin in AT and adipocytes. The expression of ghrelin in human AT was shown to be negatively correlated with BMI and followed the BMI distribution of fasting circulating ghrelin. The highest protein expression of ghrelin was found in Abd Sc AT. Furthermore, this study shows that des-acetylated ghrelin inhibits HSL production and significantly reduces glycerol release and that both forms of ghrelin influence LPL secretion, consistent with a pro-adipogenic effect of ghrelin on peripheral metabolism which is isoform dependent.

Total ghrelin serum levels were also reduced with increasing BMI. This finding is supported by previous observations (Purnell, 2003, Vendrell, 2004). In addition to this, data from this study illustrated that the same pattern of BMI alteration applies for WAT-derived ghrelin, which may reflect a physiological role of ghrelin for fat preservation as a response to the lower fat mass of lean subjects. Higher ghrelin levels in lean subjects could give rise to increased appetite stimulation of lean individuals, as supported by the ability of circulating ghrelin capable of entering the BBB (Banks, 2002) and by the reflection of the same negative correlation of ghrelin with BMI in the CSF (Tritos, 2003), similar to serum. It remains to be shown whether AT derived ghrelin can take part in a central feedback mechanism, which would in the first instance require AT-derived ghrelin to elicit endocrine activity by entering the blood stream. Taking into consideration that the preprandial rise and acute postprandial suppression of ghrelin accounts for less than 60 % of the overall ghrelin levels (Cummings, 2001; English, 2002), a considerate proportion of ghrelin secretion remains as a basal layer devoid of the influence of short term

regulation (Figure 5.4). Thus, it is possible that this baseline secretion may be supplied by organs such as AT, in the addition to the GI tract, which requires further study. The highest protein expression of ghrelin was in the Abd Sc AT depot, whereas the lowest expression was in the thigh AT depot; which is considered the metabolically least active depot in comparison to Abd Sc and omental fat (Tan, 2004). Furthermore, recent evidence suggests that the gluteofemoral depot is not associated with metabolic risk (Van Pelt, 2005).

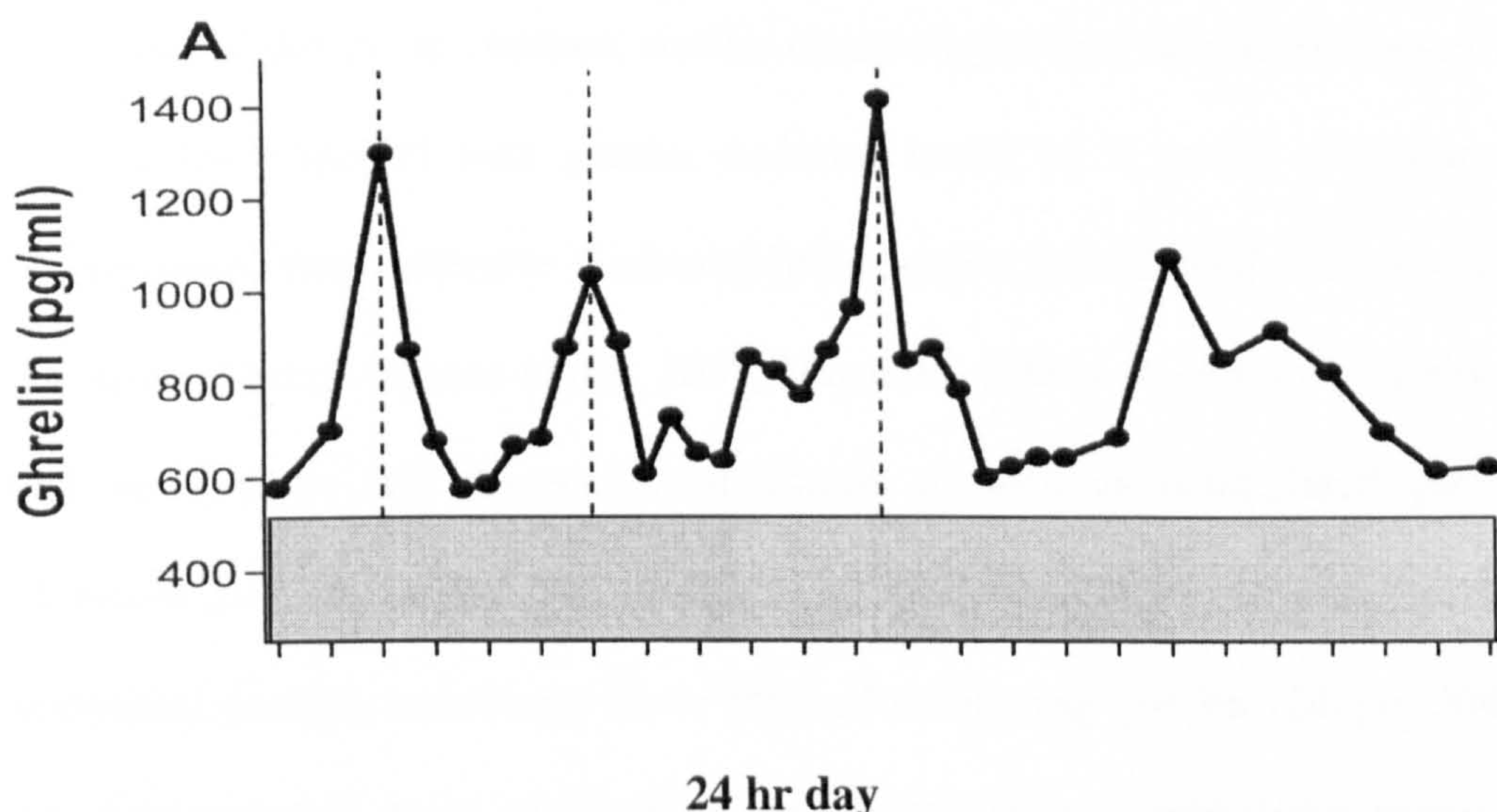


Figure 5.4.: Example of ghrelin's variation over 24hr in one subject and the three peaks represent the preprandial rise with breakfast, lunch and dinner (Cummings, 2001). The grey area under the curve represents the baseline levels of ghrelin.

Analysis of total ghrelin serum levels showed higher levels in women, which is supported by previous reports (Greenman, 2004; Akamizu, 2004). This study showed no age correlation of total serum fasting levels, which has been highlighted previously (Vilarassa, 2005; Langenberg, 2005). Interestingly, whilst ghrelin gene expression in rodents was reported to be age dependent (Liu, 2002), the age correlation of mRNA expression did not translate to the protein secretion of ghrelin. However, some authors found a positive age correlation of fasting ghrelin (Purnell, 2003), whilst others demonstrated a

negative age correlation (Broglio, 2003; Schutte, 2007). Reasons for such contradicting reports may partly be explained by unidentified cofounding factors, *e.g.*, the older population may considerably differ with regards to overall health, co-morbidity, use of medication, sex hormone status and other hormones, most of which naturally decline with age.

The results of this chapter confirm a significant effect of des-acetylated ghrelin on the level of glycerol release and HSL in human adipocytes and not octanylated ghrelin. In contrast, studies examining rodent adipocytes report an antilipolytic effect of both ghrelin isoforms based on a partial reduction of isoproterenol (non selective ß-adrenoceptor agonist) stimulated lipolysis, and not basal glycerol release (Choi, 2003; Muccioli, 2004). In addition, results of this study show that des-acetylated ghrelin as well as octanylated ghrelin influence LPL secretion. The predominant peripheral form of ghrelin, des-acetylated ghrelin, constitutes 80 to 90% of circulating ghrelin (Ghigo, 2005) and demonstrated more of an effect on adipose tissue metabolism; therefore may have a greater net-influence on overall fat mass. The two ghrelin isoforms have previously been shown to be antagonistic, with adverse influence on insulin sensitivity (Broglio, 2004). An antagonistic effect was however, not observed for lipid metabolism in this study.

The ghrelin receptor GHS-R has not yet been identified in human AT. Furthermore, mRNA expression of GHS-R has been reported as negative for human WAT (Gnanapavan, 2002). There are only few reports of GHS-R mRNA isolation in rat AT (Kojima, 1999) and rat adipocytes (Choi, 2003), which has not been confirmed by others (Muccioli, 2004). Since des-acetylated ghrelin does not bind to GHS-R (Broglio, 2004), it has been suggested that the

antilipolytic effect of ghrelin could be mediated by unidentified non-GHS-R1a receptors (Thompson, 2004). Whilst studies using *in vivo icv* injections of ghrelin in rats suggest that ghrelin is a central ligand of Y1 receptors through which it can affect appetite (Shintani, 2001) and colon motility (Tebbe, 2005), it is not known whether ghrelin is also a ligand of Y1, the most characterised receptor for NPY in human WAT.

As a result of the experiments in Chapter 4, it is evident that Y1 is expressed in all major depots of human WAT. Labelle (1997) demonstrated that the receptor Y1 mediates the antilipolytic effect of PYY and NPY in rat adipocytes; additionally, treatment of rats with antagonists of Y1 has been shown to ameliorate weight gain through reduction of appetite as well as adipocyte cell size (Ishihara, 2002). Y1 does not only bind the N-terminal end of NPY (1-36) but it has previously been shown that it can bind other ligands of the pancreatic polypeptide family with potentially higher binding affinities for PYY (1-36) and lower affinity of PP (Sylte, 1999). This study investigated whether blockade of the Y1 receptor altered the effects of ghrelin adipocyte metabolism and further investigated Y1 as potential receptor for ghrelin. Results showed that blockade of the Y1 receptor with BP3226 inhibited LPL release, with no effect on HSL secretion and that the effect on LPL and the use of the blocker were similar for both acetylated and des-acetylated ghrelin. Whilst the antilipolytic action of PYY and NPY in human adipocytes has previously been shown to be mediated by Y1, and subsequently blocked by BP3226 (Serradeil-Le Gal, 2000), the antilipolytic effect of ghrelin was not significantly altered by addition of the Y1 blocker. Thus the effect of ghrelin does not appear to be mediated by Y1. Whilst such results are in agreement with previous reports

from rat physiology, which suggest that both forms of ghrelin stimulate LPL release (Thompson, 2004), the adipogenic effect of ghrelin was blocked by the use of BP3226, suggesting that both ghrelin isoforms may in fact be a ligand of Y1 for mediation of its lipogenic effect in humans. Ultimately, distinction of the individual effects of the two ghrelin isoforms requires further *in vivo* analysis.

In summary, this study found protein expression of ghrelin in human adipocytes; furthermore its expression is negatively associated with BMI, similar to circulating ghrelin and previously reported CSF ghrelin. The autocrine effect of ghrelin in human WAT, with an antilipolytic and lipogenic effect, appears to be aimed at fat preservation and shows differences with rodent physiology, based on the observation that des-acetylated ghrelin is able to directly influence glycerol release. Whilst the antilipolytic effect of ghrelin is not affected by Y1, which mediates the antilipolytic effect of PYY and NPY, the adipogenic action of ghrelin appears to be influenced by Y1 blockade and shared by both ghrelin isoforms, as they both elicit a similar effect on lipogenesis. Finally, the receptor responsible for the lipolytic action of ghrelin in WAT has yet to be identified.

Chapter 6

CHRNA and adipose tissue

6.1. Introduction

Smoking influences appetite and reduces food intake by its addictive component, nicotine (Li, 2000; Jo, 2005). The resulting reduced body weight (Grunberg, 1985) and post-cessation weight gain is not confined to humans (Klesges, 1989), but also seen in rodents (Zhang, 2001), which suggest physiological responses to nicotine. Whilst nicotine can influence several pathways involved in energy regulation (Figure 6.1.), nicotine also modulates NPY expression in the hypothalamus (Li, 2000; Frankish, 1995).

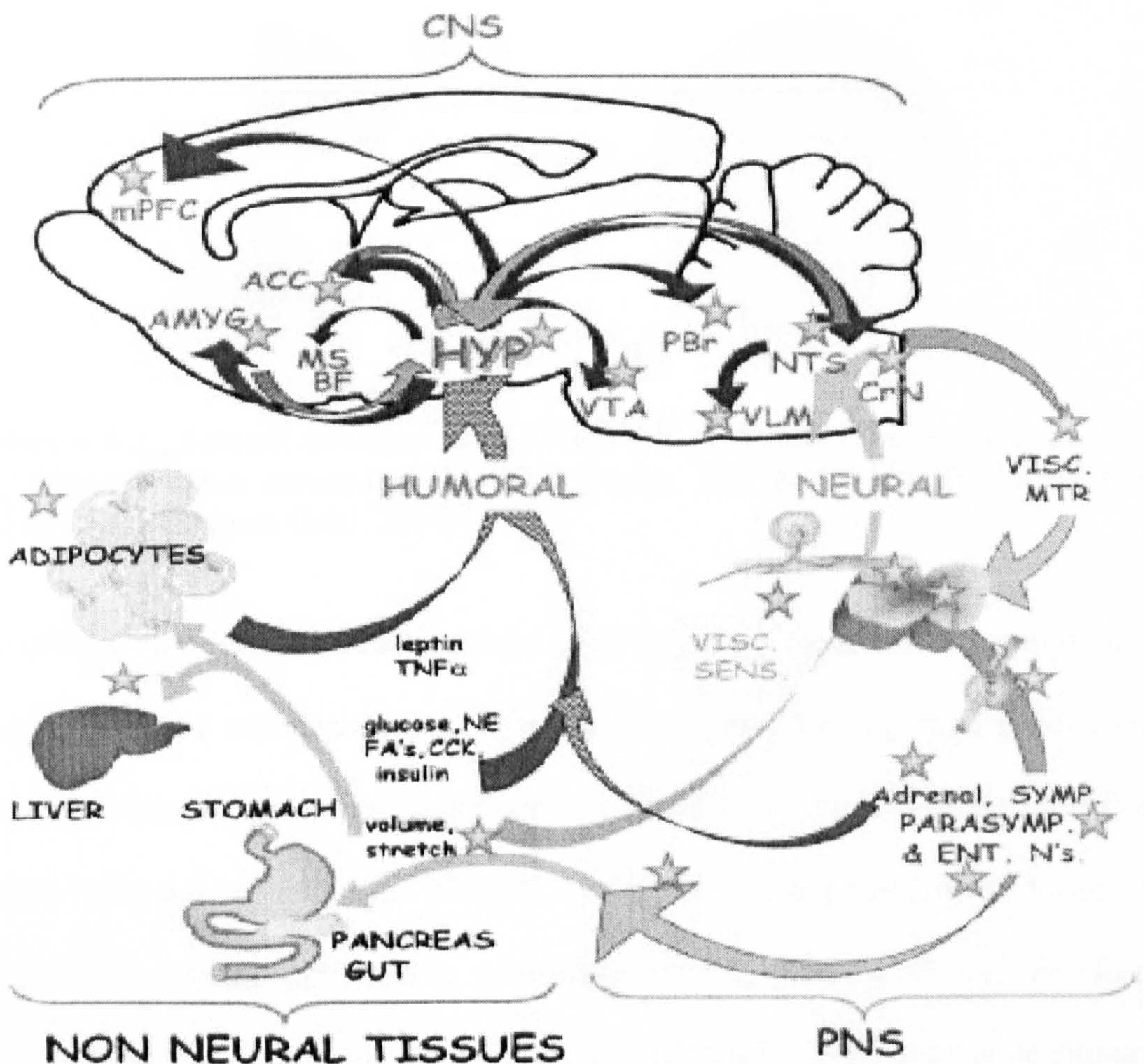
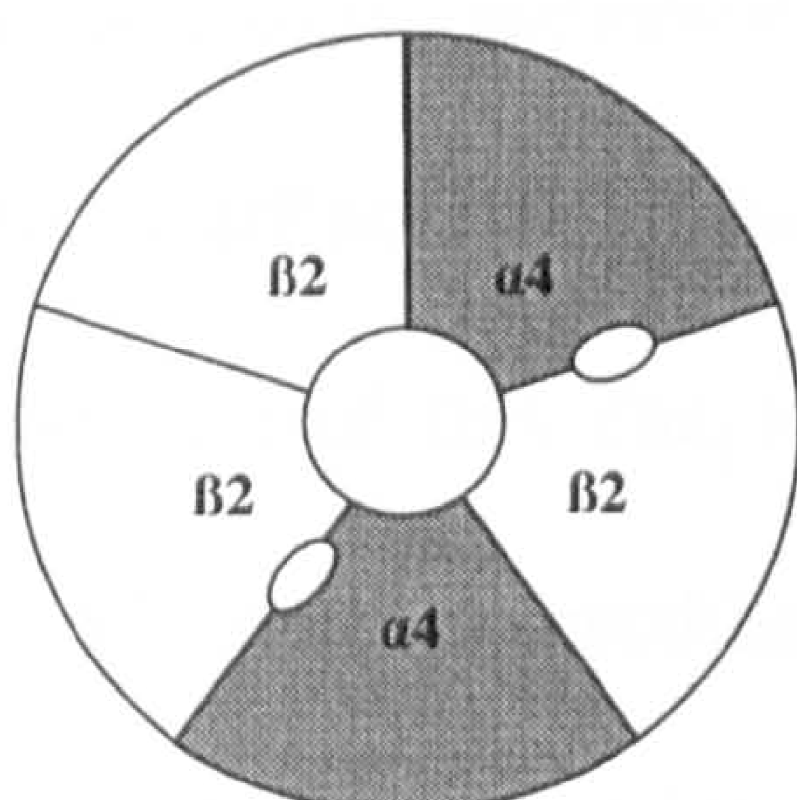


Figure 6.1.: Multiple pathways through which nicotine affects appetite. PNS: peripheral nervous system. Stars signify sites of nicotine action (Adapted from Jo, 2002).

The peripheral and central actions of nicotine are mediated by upregulation of the cholinergic nicotinic receptors (CHRNAs) (Peng, 1997; Ke, 1998) which are responsible for smoking addiction (Kelley, 2002) and appetite regulation (Jo, 2002). Whilst chronic nicotine exposure is associated with a redistribution of body fat (Jensen, 1995) and enhanced lipolysis in rodents and humans (Sztalryd, 1996; Andersson, 2001), little is known about the mechanism by which nicotine influences fat mass.

Heteromeric receptor CHRNA4 **Homomeric receptor CHRNA7**



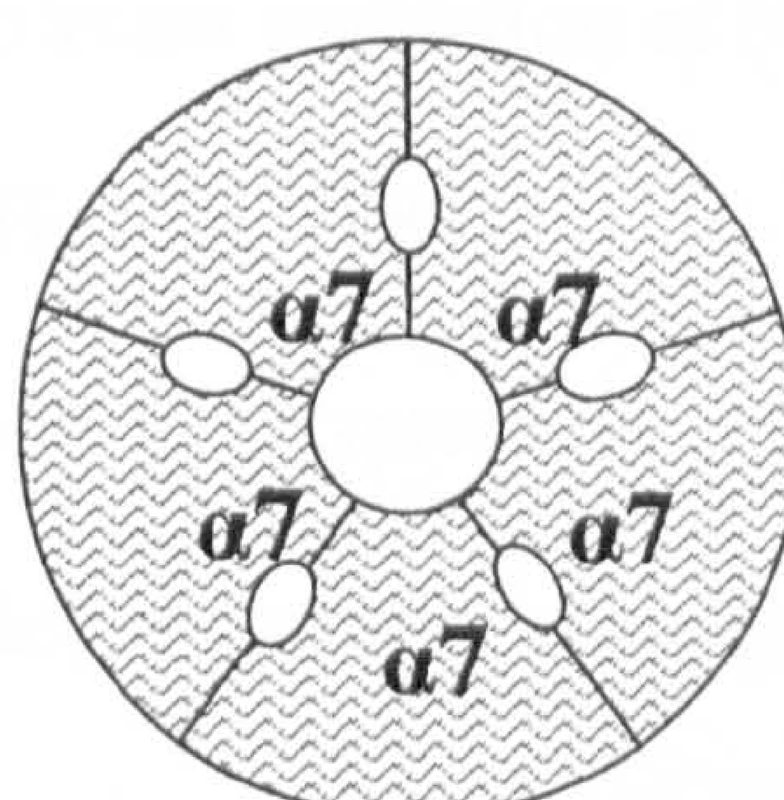


Figure 6.2.: Subunit arrangement of CHRNA4 (consisting of α4ß2 subunits) and CHRNA7, which consists of five α7 subunits. The circles signify the binding sites (Adapted form Gotti, 2004)

CHRNAs are pentameric (consistent of 5 subunits) receptors composed of a selection of 12 subunits (nine α- and three ß-subunits) for which 12 genes were cloned. Nicotinic receptors can be further divided into subfamilies, depending on their type of subunits (Le Novere, 1995). The two predominant forms of CHRNAs are the heteromeric constellation α4ß2 (CHRNA4) and the form containing five homomeric subunits of α7 (CHRNA7). CHRNAs can be situated presynaptic, where they modulate neurotransmitter release; or postsynaptic, where they take part in signal transduction or perisynaptic, where they take on diverse modulatory functions (Berg, 2002). Since both of these receptors are associated with smoking addiction, they have become targets for drug

development of antismoking agents, *e.g.*, Vanrenicline (Yates, 1995; Mihalak, 2006). Although these receptors are typically neural receptors which exist in the central and peripheral nervous system, forms of these heterogeneous receptors have recently been discovered in non-neuronal cells, e.g., muscle, skin, lung and immune cells (Gotti, 2004). The function of these receptors in the individual organs/cell types is not fully established (Gotti, 2004). It has not yet been determined whether CHRNAs are expressed in AT, where they may influence adipogenesis by modulation of neurotransmitter release, *e.g.*, noradrenaline and NPY, similar to the previously reported downstream regulation of NPY secretion in the chromaffine cells of the adrenal gland (Hexum, 1989).

Thus, the aim of this chapter was to examine the expression of CHRNAs in human AT and to establish a possible depot-specific distribution and to determine whether smoking affects circulating levels of NPY in serum, which could be potentially associated with CHRNA regulation in AT.

6.2. Methods

6.2.1. Subjects

Adipose tissue (AT) samples were obtained from thirty eight consenting Caucasian women undergoing elective surgery. Mean age: 42.7±7.3(mean±SD) years; BMI: 26.2±4.5kg/m^2 Abdominal subcutaneous (Ab Sc), omental (Om) AT and thigh tissue (Th) AT samples were collected with the approval of the South Birmingham Ethics Committee. Subjects on endocrine therapy (e.g., steroids, HRT, thyroxine) and patients with malignant diseases were excluded. AT samples were immediately processed for adipocyte isolation and cell culture or flash frozen for protein or RNA extraction as described below.

Fasting serum samples of 34 Caucasian healthy volunteers (14 men and 20 women) were taken with approval of the Coventry and Warwickshire ethics committee. Characteristics of these subjects were: age: 48.7±14years, BMI: 26.2±4.5kg/m^2, all subjects are Caucasian and not treated with any medication or hormone replacements. Non-smokers were defined as subjects who never smoked. Ex-smokers were thus not included. Freshly obtained serum samples were centrifuged at 700 x g for 20 min, snap frozen and stored at -80°C. Serum was used for NPY analysis.

6.2.2. Adipocyte isolation:

Adipose tissue was processed as previously described in Chapter 2 and washed with 1X Hank's balanced salt solution containing penicillin (100U/mL) and streptomycin (100µg/mL). The tissue was finely cut, digested with collagenase class 1 (2mg/mL, Worthington Biochemical Corporation, USA) in 1X Hank's

balanced salt solution (Gibco, USA) for 1 hr at 37°C in a water bath, then shaken at 100 cycles/min at 37°C. Following digestion, the tissue was filtered through a sterile double-layered cotton mesh and adipocytes were separated by centrifugation at 360 x *g* for 5 min. The upper layer of mature adipocytes was removed from the collagenase-dispersed preparation, washed twice in phenol red-free medium DMEM: F12 and centrifuged at 360 x *g* for 2 min. Adipocytes were cultured in flasks ($25cm^2$) in phenol red-free Dulbecco's modified Eagle medium (DMEM/F-12) medium containing glucose (15mmol/L), penicillin (100 U/mL), and streptomycin (100µg/mL). Aliquots of 1mL containing ~500,000 mature adipocytes were maintained in medium (5mL per 25-cm^2 flask) for 48 hrs. After incubation of adipocytes (37°C/5%CO_2), the conditioned media and adipocytes were separated by centrifugation (360 x *g* for 2 min) and the media removed.

6.2.3. Western blot analysis:

Homogenised human AT was extracted using a 10% RIPA buffer method (see Chapter 2.3.). Protein concentrations were determined using the Bio-Rad Detergent Compatible (DC) protein assay kit (Biorad UK). Western blot analysis was performed using a method described in Chapter 2.4.. In brief, 20µg/lane of adipocyte protein was separated by SDS-PAGE gel electrophoresis using a 12% gel, then electrophoresed at 120V for 1hr. Samples were electroblotted in a vertical transfer apparatus at 100V for 1hr. Membranes were blocked overnight at 4°C in phosphate buffered saline and Tween 20 (PBS-T: PBS + 0.05% Tween 20; Sigma, UK) containing 10% (w/v) non-fat milk powder (Marvel, Moreton, Merseyside, UK). Membranes were incubated for 1 hr with primary polyclonal

antibody CHRNA7 (Abcam, Cambridge, UK) 1:2000, then in conjugated anti-rabbit HRP secondary antibody in concentrations of 1:20,000 diluted in PBS with 0.5%Tween. CHRNA7 (56kDa) was detected by chemiluminescent assay ECL (Amersham, Little Chalfont, UK), which enabled visualization after exposure to X-ray film and the band intensity was determined by densitometry. Equal loading was ensured with use of the alpha tubulin antibody (Abcam, Cambridge, UK).

6.2.4. RNA extraction

RNA was extracted from adipose tissue and adipocytes (n=38; age: 40.9±96years; BMI: 24.9±2.8kg/m^2) taken from abdominal subcutaneous (n=30) and thigh (n=8) depots, using a RNeasy Lipid Tissue Mini Kit (Quiagen, Crawley, UK). Genomic DNA was removed using a DNase digestion step to avoid contamination and quantified using the Nanodrop ND-1000 Spectrophotometer (LabTech, UK). 1μg of RNA from each sample was reverse transcribed (RT) using AMV reverse transcriptase (Promega, Southampton, UK) according to the manufacturers' instructions.

6.2.5. Real time PCR

RT-PCR was performed in a reaction mix containing TaqMan® Universal PCR Master Mix (AmpErase UNG), added to an assay mix containing 900nM PCR primers, 250nM FAM™ dye-labelled TaqMan® MGB probe and 50ng cDNA. All reactions were multiplexed with the housekeeping gene 18S (Applera, UK), provided as a pre-optimised control probe. Messenger RNA levels were determined using an ABI 7700 real time PCR Sequence Detection system. Data

were obtained as Ct values according to the manufacturer's guidelines. ΔCt values were determined with the formula: ΔCt = Ct of the target gene minus Ct of the housekeeping gene. Measurements were repeated at least three times for each sample. Reactions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min. Primers and probes were obtained for CHRNA4 (ABI Taqman® Gene Expression Assay: Hs00181247_m1) and CHRNA7 (ABI Taqman® Gene Expression Assay: Hs00608028_m1).

6.2.6. NPY ELISA

Adipocyte derived conditioned media were concentrated 10 times through lyophilisation with the centrifuge evaporator and reconstituted in assay buffer. These samples and serum samples of smokers and non-smokers were analysed using the commercially available NPY ELISA kit (Peninsula Laboratories Inc., USA). Sensitivity: 0.04-0.06ng/mL; Intra-assay variation: <5%; Inter-assay variation: <14%.

6.2.7. Statistics

Gene expression was compared using the ΔΔCt method and statistics performed at the ΔCt stage. Statistical analysis was performed using the Student's t-test unless otherwise stated. The threshold for significance was $p<0.05$. Data in the text and figures are presented as mean ± SEM unless otherwise stated.

6.3. Results

6.3.1. mRNA expression of CHRNAs

Adipose tissue and isolated adipocytes were assessed for mRNA expression of CHRNA4 and 7, using Real-time PCR, as described previously. No CHRNA4 mRNA expression was identified in either AT or isolated adipocytes. In contrast, we detected CHRNA7 mRNA expression in human adipose tissue (AT) which was no different in AT when compared with adipocytes originating from the same patient and depot (Figure 6.3.1.1.).

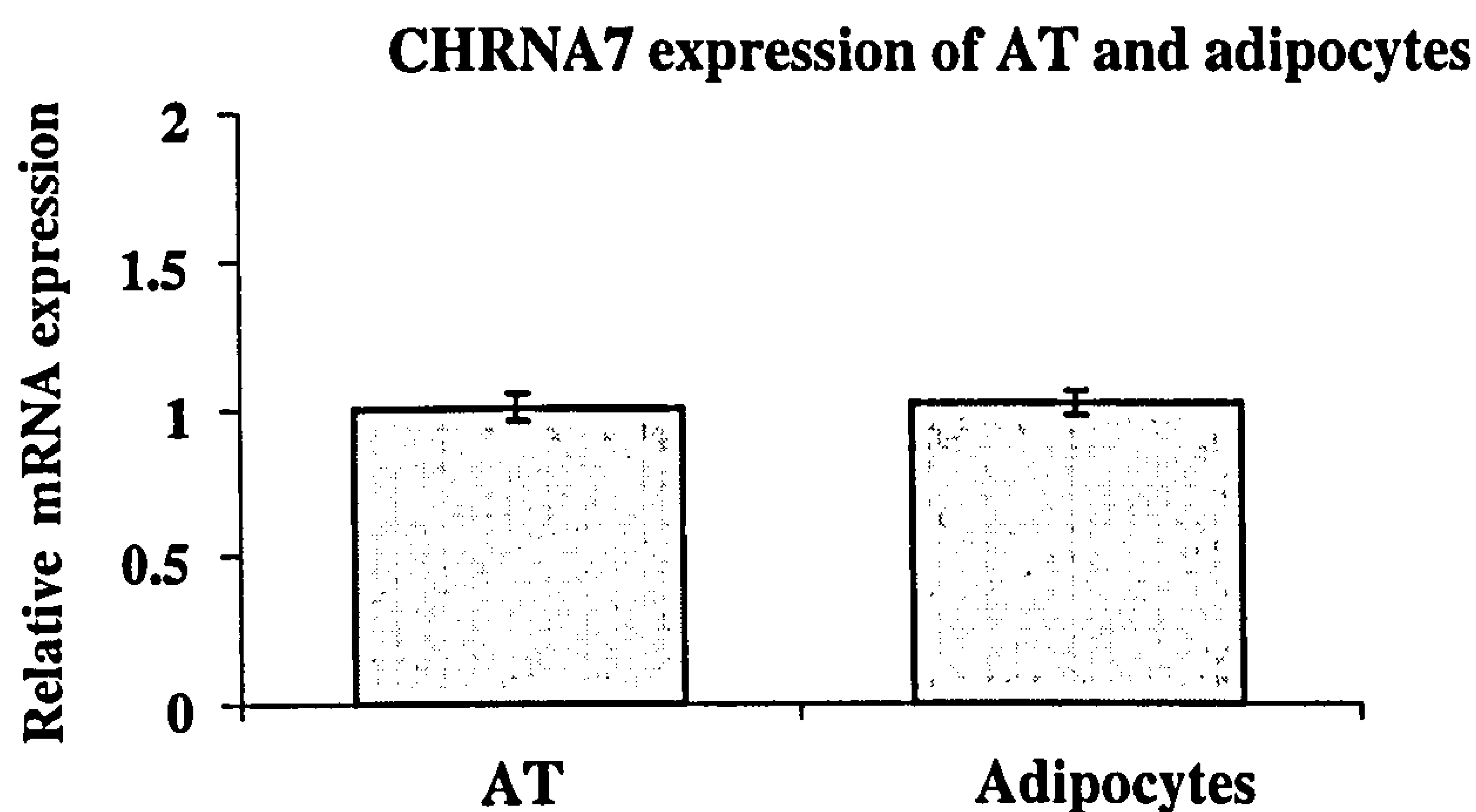


Figure 6.3.1.1.: Relative expression of CHRNA7 in human adipose tissue (allocated value of 1±SEM) in comparison with human adipocytes. Samples belong to the same depot and patients, n=8.

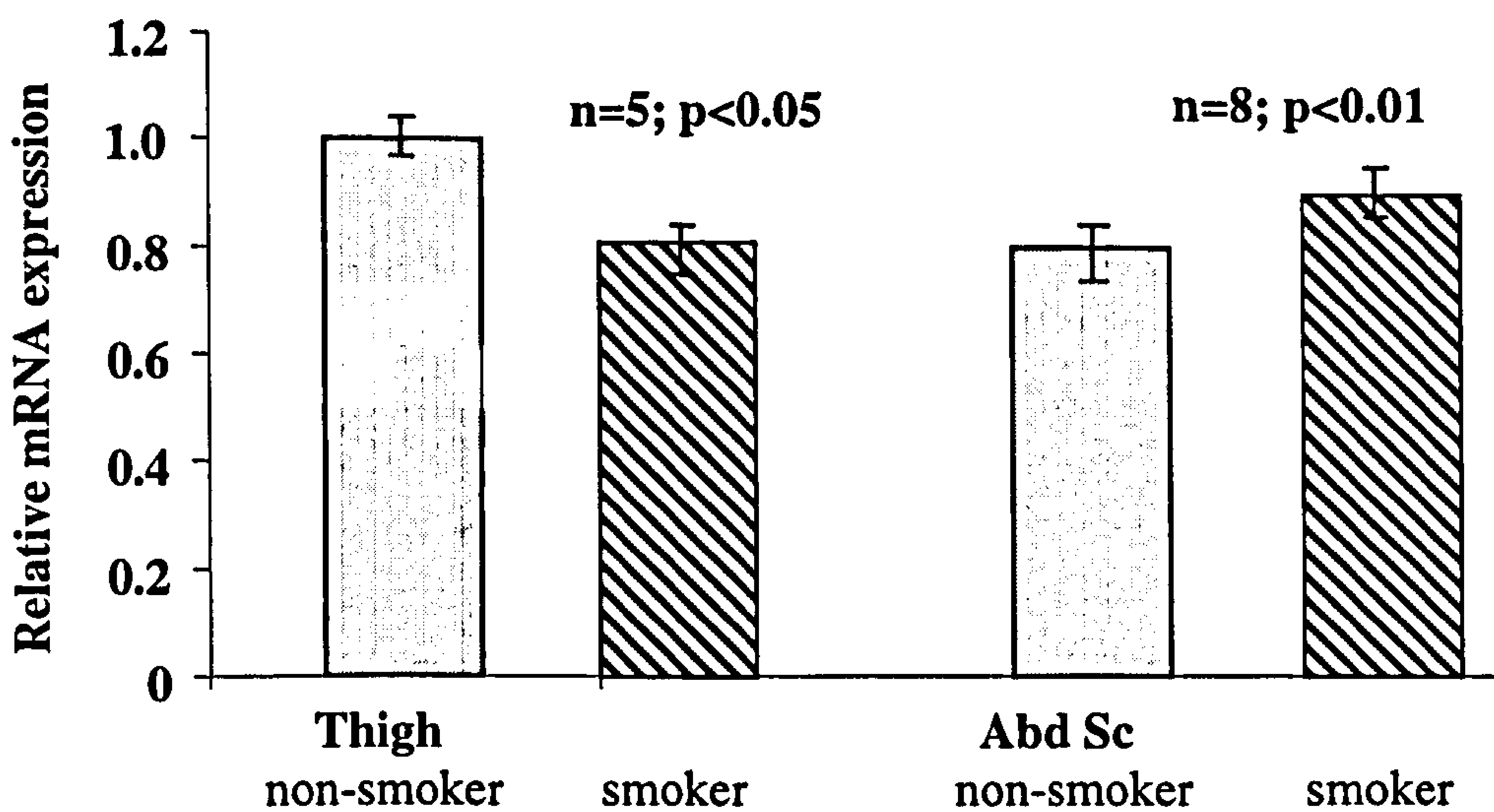


Figure 6.3.2: The graph indicates relative mRNA expression of CHRNA7 comparing Abd Sc and thigh AT depots. A value of 1 was arbitrary allocated to the expression of thigh fat in non-smokers. There was a significantly higher CHRN7 expression in thigh sc AT (n=5 matched samples) in non-smokers than smokers, with an opposite pattern in Abd Sc AT; n=8).

The comparison of the thigh and Abd Sc AT fat depot showed a higher expression in thigh fat (1±0.02OD), for which an arbitrary value of 1 was allocated. A significantly lower expression was found in age and BMI matched smokers (0.8±0.03OD) in comparison to non-smokers. In contrast, the comparison the Abd Sc AT showed a significantly higher expression of CHRNA7 in smokers (0.9±0.03OD versus 0.79±0.05OD; $p<0.01$) (Figure 6.3.1.2).

6.3.2. CHRNA protein expression

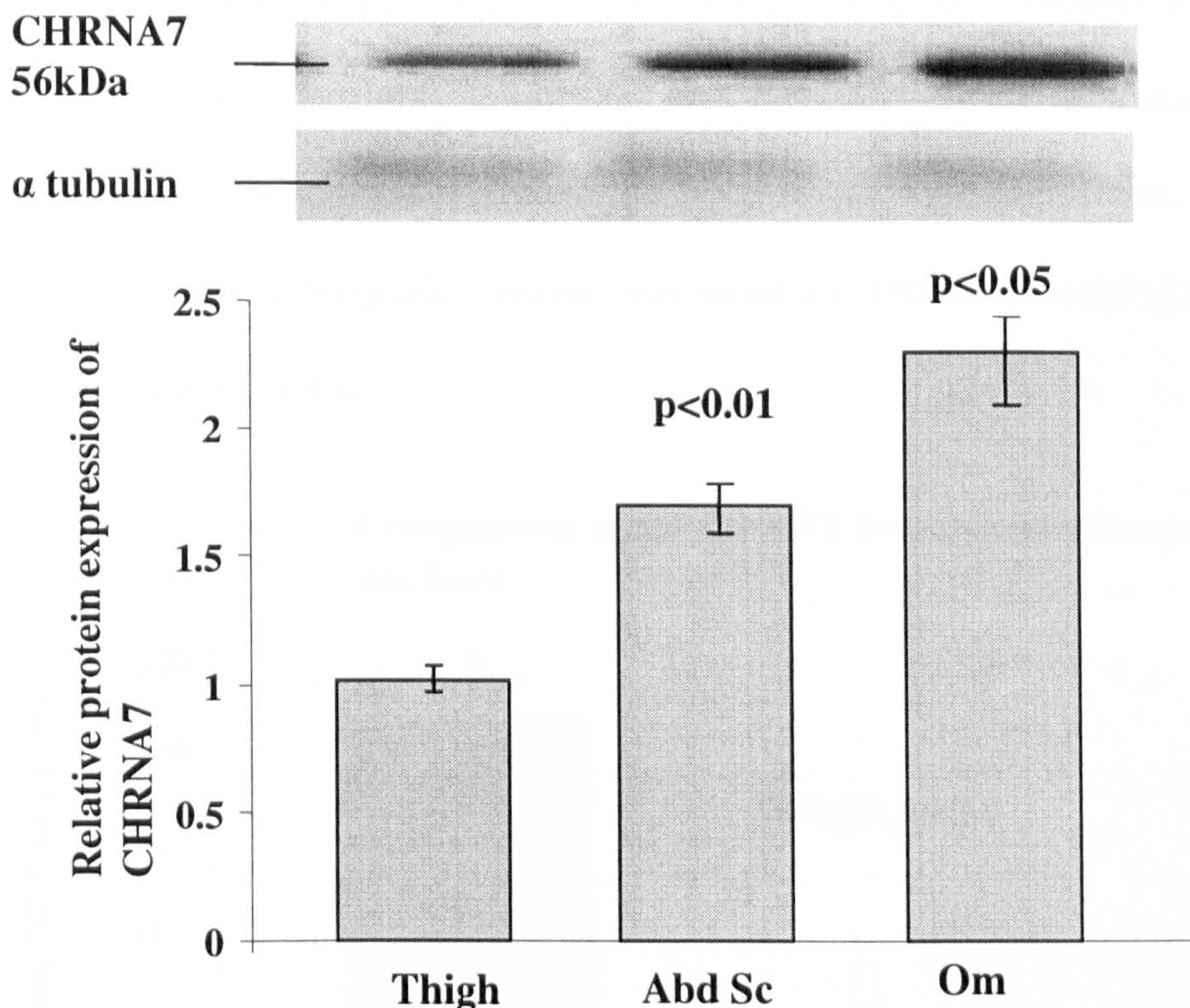


Figure 6.3.2.: Protein expression and representative Western blot of CHRNA7 expression in human fat depots with reference of thigh as (1±SEM). There is higher CHRNA7 expression in the Abd Sc AT depot ($p<0.01$) in comparison to thigh AT; and higher expression in the Om in comparison to Abd Sc AT ($p<0.05$).

CHRNA7 protein expression was further examined by Western blot. CHRNA7 is a 56kDa protein, which is present in Sc thigh, Abd Sc AT and omental fat. Semi-quantitative comparison with arbitrary allocation of the value of 1 for the expression in thigh fat (1±0.045) (relative value in comparison to thigh AT of the same subject) showed significantly higher values in Sc abdominal AT (1.7± 0.14,$p<0.01$), with highest levels in omental fat (2.3±0.28, p<0,05) of non-smokers (Figure 6.3.2). A comparison of Abd Sc AT CHRNA7 expression of non-smokers did not, however, show a BMI correlation (BMI of obese subjects 32.5±2.6kg/m^2 compared with lean subjects 23.2±1.2kg/m^2, n=8, p=NS).

6.3.3. Serum NPY levels of smokers versus non-smokers

We analysed serum levels of 34 Caucasian subjects (20 women, 14 men, age 48.7±14yrs; BMI 26.2±4.5kg/m^2) consisting of 16 current smokers and 18 non-smokers, which were matched by age and BMI. Correcting for these variables with stepwise regression analyses, the NPY levels were significantly lower in smokers (34.3±1.54ng/mL) versus non-smokers (103.8±16.4ng/mL, p=0.024; n=35) (Figure 6.3.3.).

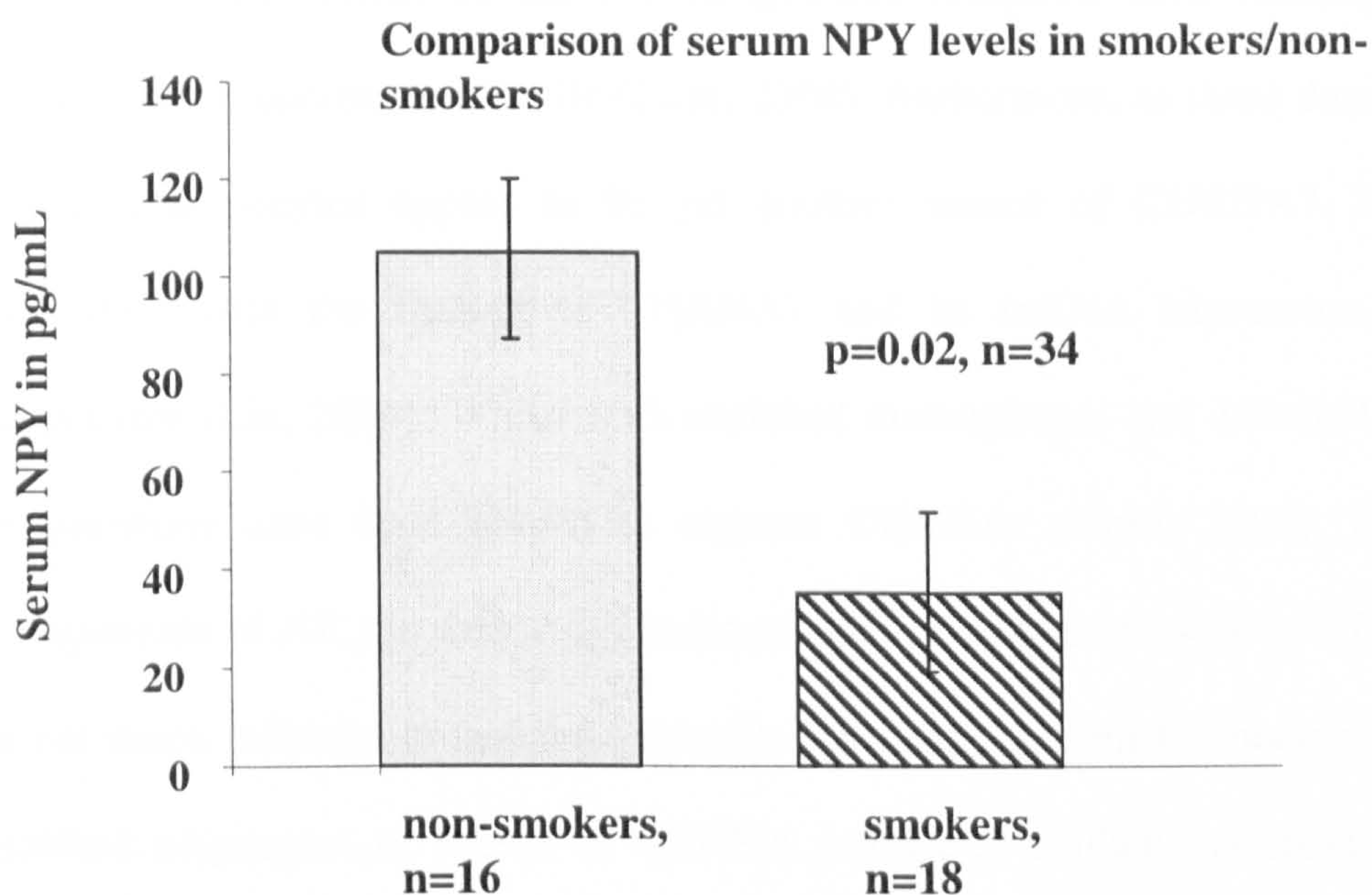


Figure 6.3.3: Fasting serum NPY levels compared between age and BMI matched smokers and non-smokers.

6.4. Discussion

Nicotine is an exogenous ligand for CHRNA receptors, where it competes with the endogenous ligand acetylcholine (Berg, 2002). The ability of nicotine to bind to CHRNAs has made smoking and smoking-related pathologies convenient models for the study of these receptor types. This is the first study to demonstrate CHRNA7 receptor mRNA and protein expression in human fat. Although these receptors are typically neural receptors which exist in the central and peripheral nervous system, forms of these heterogeneous receptors have recently been discovered in non-neuronal cells (Gotti, 2004); furthermore, as these data show, AT and adipocytes appear to be yet another source of CHRNA7. This is consistent with the finding of CHRNA7 and its mRNA expression in rat adipocytes (Liu, 2004). Whilst both enriched macrophages and endothelial cell preparations have been shown to express CHRNAs (Gotti, 2004), and are components of AT, the data above indicates that mRNA expression of CHRNA7 is not much different in isolated adipocytes themselves, whilst contamination of isolated adipocytes as source of CHRNA mRNA is unlikely, as discussed in Chapter 4. The presence of CHRNAs in AT is further supported by studies examining the effect of smoking on AT. Nicotine induces lipolysis, as demonstrated by differences in serum and adipocyte glycerol secretion after nicotine infusion in rodents (Sztalryd, 1996) or with smoking in humans (Andersson, 2001). Pre-treatment with the nicotinic receptor antagonist mecamylamine can abolish this lipolytic effect of nicotine, as demonstrated by microdialysis studies (Andersson, 1995).

Furthermore, this study identified a depot-specific expression of CHRNA7 in AT, which could partially be responsible for the fat distribution with smoking.

Body fat distribution in chronic smokers is characterised by an increase in abdominal fat and a decrease of gluteal/thigh fat (Jensen, 1995), and a reversal of this distribution has been documented with smoking cessation in which unhealthy visceral fat diminishes and gluteofemoral fat increases (Ferrara, 2001). Whilst CHRNA7 protein appears to be generally more highly expressed in abdominal fat, similar to previously described sympathetic innervation (Hoffstedt, 1997), its mRNA expression is higher in the Abd Sc fat of smokers in comparison to non-smokers, which could have been upregulated in smokers by exposure to nicotine. The above experiments do not show a correlation of CHRNA7 protein expression with BMI in AT, which may be due to the fact that BMI does not reflect the depot-specific distribution of fat.

The correlation of circulating NPY levels with smoking is in support of a link between CHRNA7 and NPY metabolism. A variety of neurotransmitters can be controlled by CHRNA7, e.g. amongst others, it modulates glutamate release in the hippocampus, GABA and acetylcholine (Berg, 2002). NPY release can also be modulated by nicotinic receptors in the adrenal gland (Hexum, 1989). Furthermore, CHRNA7 can participate in both: sympathetic and parasympathetic pathways. Further research is required to demonstrate a direct relationship between CHRNA7 and NPY release in Abd Sc adipocytes which could be done by cell treatment with selective CHRNA7 antagonists and measurement of subsequent changes in NPY secretion. Further analysis is also required to determine the influence of CHRNA7 on adipokines, which are known to be affected by nicotine exposure (Nicklas, 1999; Arai, 2001; Liu, 2004). The reduced serum NPY levels in smokers may further reduce the orexigenic

hypothalamic effects of NPY, in addition to the hypothalamic downregulation of NPY observed by Frankish (1995), and affect central appetite regulation through reduced food intake as result of nicotine exposure (Li, 2000). A crosstalk of peripheral circulating NPY with the hypothalamus is likely, given its easy entry into the CSF, as observed by the study of Kastin (1999), in which radioactive labelled NPY entered the BBB in an unsaturated manner, faster than albumin and similar to diffusion. NPY mediates orexigenic signals through NPY receptor 1 (Y1), Y4 and Y5, whilst Y2 is an auto-inhibitory receptor which mediates appetite inhibition (Broberger, 1997). The anorexigenic gut hormone PYY, which belongs to the same family of pancreatic polypeptides (with 70% sequence homology with NPY), has also high affinity to the receptor Y2, through which it is thought to suppress appetite (Abbott, 2005). A potential difference in binding affinities of NPY and PYY to hypothalamic human Y2 has not yet been clarified; however, competitive binding of PYY and NPY for Y2 is possible. Whilst NPY is suppressed by smoking, PYY action may therefore prevail. Furthermore, nicotine affects the gastrointestinal PYY mRNA expression in rats (Gomez, 1996), little is known about the influence of nicotine exposure on cerebral levels of PYY, which can also cross the BBB (Nonaka, 2003).

Whilst CHRNA may be responsible for the net decrease of circulating NPY levels, it may modulate NPY action in fat in a depot specific manner. Several studies using human adipocytes show an antilipolytic effect of NPY (Castan, 1994; Turtzo, 2001); this was confirmed in Chapter 4, demonstrating a decrease of glycerol secretion as a result of rh NPY treatment of isolated cultured adipocytes. The increased waist-hip ratio in smokers may be induced by the

antilipolytic effect of NPY which shows higher expression in Abd Sc fat as shown in Chapter 4, and therefore requires further study.

In summary, CHRNA 7, but not 4, is expressed by human adipocytes in a depot specific manner and appears to be regulated by smoking. The pattern of CHRNA7 fat distribution is highest in visceral fat, in which sympathetic innervation is predominant. Circulating NPY levels are reduced in chronic smokers, which may in part contribute to the reduced orexigenic hypothalamic NPY levels and lead to reduced food intake. Whilst this study suggests that the antilipolytic neurotransmitter NPY is associated with smoking, which may explain the smoking related fat redistribution, further research is required to demonstrate the signalling mechanisms of CHRNA7 in AT and the pathway by which it influences NPY.

Chapter 7

Discussion

Adipose tissue, which is now widely recognised as an endocrine organ, influences and interacts with the brain (Mohamed-Ali, 1998). The secretion of adipokines enables AT to participate in overall energy regulation. The discovery of the adipokine leptin and its hypothalamic action provided the first example of AT crosstalk with the brain (Baura, 1993). As an indicator of overall energy status, leptin can deliver feedback to the brain and regulate appetite through hypothalamic pathways. Furthermore, leptin can regulate adipogenesis by central autonomic feedback (Rayner, 2001) to AT and by autocrine/paracrine activity in AT. Meanwhile, the existence of other adipokines has been reported with yearly acquisition of new proteins to the list, some of which, *e.g.*, nesfatin (Oh-I, 2006) have been discovered recently, and have been shown to interact with the brain. Alternatively, evidence of adiponectin and resistin interaction with the human brain is lacking. In light of a 'brain-to-adipose system' crosstalk, it has been suggested that central neurotransmitters may themselves be expressed in AT (Yang, 2003), whereby they may potentially alter fat metabolism by paracrine and autocrine mechanisms, to elicit feedback on energy homeostasis.

The projects of this thesis established the importance of further proteins and their potential signalling pathways in the crosstalk between AT and the brain in human physiology. The studies examined the regulation of energy homeostasis using a two sided approach. Firstly, the crosstalk between AT and the brain by CNS action of adipokines was studied. Secondly, mirroring this, the CNS-AT crosstalk by expression of neurotransmitter and their receptors in AT was explored. The adipokines adiponectin and resistin were determined to have the potential to

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Chapter 7

Discussion

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APPENDIX I: Chemicals and Solutions

A1.1. Western blotting solutions:

4% sodium dodecyl sulphate (SDS)

4g SDS powder
100ml mQH_2O or

10ml 20% SDS solution
50ml mQH_2O
Solution was stored at RT

Non reducing loading buffer
625μL Tris-HCl (pH 6.8) 125mM
500μL SDS 4%
1000μL Glycerol
125μL Bromophenol blue
250μL Distilled water

For reducing loading buffer 200μL DTT was added

Electrode buffer for SDS-PAGE electrophoresis:

REAGENT	FINAL CONCENTRATION (X5)	QUANTITY (DILUTED IN 1L)
Tris	1.24×10^{-1}M	15g
Glycine (Biorad, Hercules, USA)	9.6×10^{-1}M	72g
SDS	20%(v/v)	25ml

Table AI.1.1.: Components of electrode buffer

Electrode buffer was stored at 4°C.

Transfer buffer for electrophoresis:

REAGENT	FINAL CONCENTRATION (X1)	QUANTITY (DILUTED IN 4L)
Tris	25mM	15.15g
Glycine	192mM	72.0g
Methanol	100%	1L

Table AI.1.2.: Components of transfer buffer

The buffer was stored at 4°C in the cool room.

Phosphate buffered saline (PBS) (pH7.6)

1l of distilled water was mixed with 5 tablets of:

PBS 120mM
NaCl 2.7Mm
KCL 10mM

The solution was stored at RT.

1.0% PBS-tween (PBS-T)

1L PBS (prepared as above)
1ml Polyoxyethylene Sorbitan Monolaurate ('Tween20' (0.1% (v/v), Sigma UK)
Solution was stored at room temperature.

20% blocking solution for Millipore® filters

20g non-fat Milk solution (Marvel Milk Powder)
200ml PBS 0.5% Polyoxyethylene Sorbitan Monolaurate (Tween 20 (0.1% (v/v), Sigma UK)

0.5% PBS/PBS-T (diluents for antibodies)

2ml 1.0% PBS-T (prepared as above)
2ml PBS (prepared as above)

A1.2. Cell culture solutions:

Phosphate buffered saline (PBS) (pH7.6)

PBS 120mM
NaCl 2.7mM
KCL, 10mM
Solution was filtered and penicillin (pen) (100units/ml) and streptomycin (strept) (100mg/ml) added. Solution was stored at room temperature.

Lysis buffer

Ammonium chloride (NH_4Cl)	0.154 mol/L
Potassium bicarbonate ($KHCO_3$)	10mmol/L
Ethylenediaminetetraacetic acid (EDTA 0.5M)	0.1mmol/L

Solution was stored at room temperature.

Trypsin solution

Trypsin 2.5%	
Ethylenediaminetetraacetic acid	(0.25mM)
Phosphate buffered saline	(pH7.6)

Pre-adipocyte cell growth medium

Dulbecco's minimal essential medium (DMEM/F-12)
15% filtered foetal calf serum (FCS)
penicillin (100units/ml) and streptomycin (strept) (100mg/ml) added
Growth medium was stored at 4°C.

Phenol red free medium

Dulbecco's minimal essential medium (DMEM/F-12)
Phenol red free medium
1% transferrin (see below)
Penicillin (100units/ml) and streptomycin (strept) (100mg/ml) were added
and medium was stored at 4°C.

Transferrin

Transferrin binds Fe^{2+} and prevents its oxidisation to Fe^{3+}. Transferins are important extracellular antioxidants. The binding of iron to transferrins is very effective under physiological conditions so that virtually no free iron exists which could catalyse the production of free radicals.

AI.3. Protein extraction solution (RIPA)

RIPA buffer (10ml)

9mL	1xPBS
0.1mL	1% NonIdet P40
0.05mL	0.5% sodium deoxycholate
0.01mL	0.1% SDS
1mL	1x complete mini protease inhibitor cocktail
100µg/mL	ALLN

AI.4. Solutions for RT PCR

RNA isolation:

DNase I	REACTION BUFFER	STOP SOLUTION
1unit/µL in 50% glycerol 10mM Tris-HCl (pH7.5) 10mM $CaCl_2$ 10mM $MgCl_2$	200mM Tris-HCl (pH 8.3) 20mM $MgCl_2$	50mM EDTA

Table AI.4.: Solutions for RNA isolation

Taq DNA polymerase for visualisation of PCR products

50mM Tris-HCl (pH 8.0)
100mM NaCl
0.1mM EDTA
1mM DTT
50% glycerol
1% Triton® X-100

Reverse Transcription (RTn) Buffer

100mM Tris-HCl (pH 9.0 at 25°C)
500mM KCl
1% Triton® X-100

APPENDIX II: Methods

AII.1.Rainbow markers for protein size comparison in western blot analysis

Figure AII.1.: A Marker for medium range molecular weights (Amersham Pharmacia Biotech, Buckinghamshire, UK). The marker is resolved on a SDS-PAGE gel; after electrophoresis the gel was dried and photographed. Black bands represent the gel exposed to photographic film (Hyperfilm™ β-max) and the protein weight estimated by relating the black bands on photographic film with the colour coded bands on the membranes.

AII.2. Cell count with the use of a haemocytometer

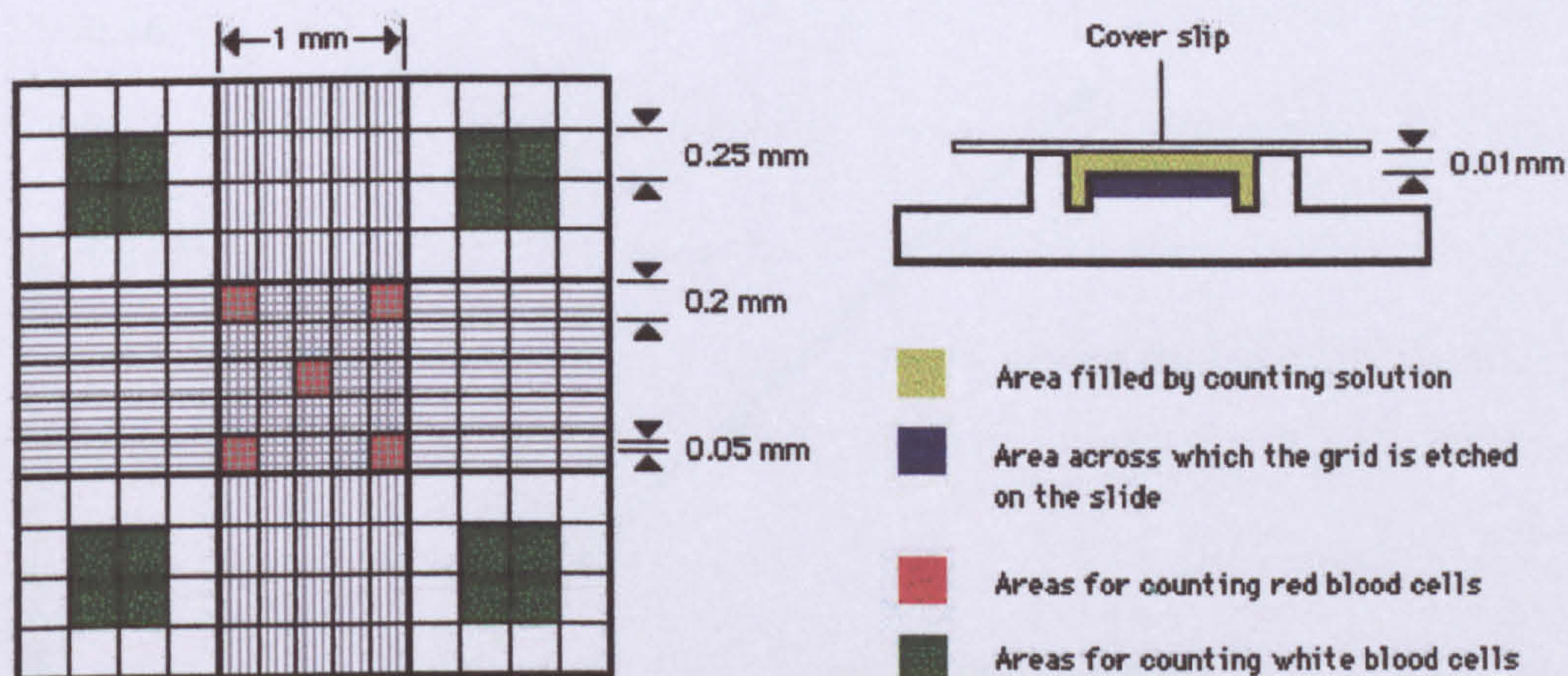

Figure AII.2.: Picture shows a standard haemocytometer and its profile in cross section.

The haemocytometer is viewed at 100x magnification using a light microscope (magnification is a multiple of 10x ocular and 10x objective).

Each square of the haemocytometer represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Cell concentrations are calculated as follows (as $1cm^3 \approx 1ml^3$):

cells per ml = the average count per square * dilution factor* 10^4 (count 10 squares)

cell viability = total viable cells (unstained)/ total cells (stained and unstained) x 100

AII.3.Calculation of protein content of samples for Western analysis

Samples were analysed on a spectrophotometer at 655nm. The optical densities were converted to protein content (µg) by the construction of a standard curve (Figure AII.3.) with known concentrations and serial dilutions of bovine serum albumin (BSA).

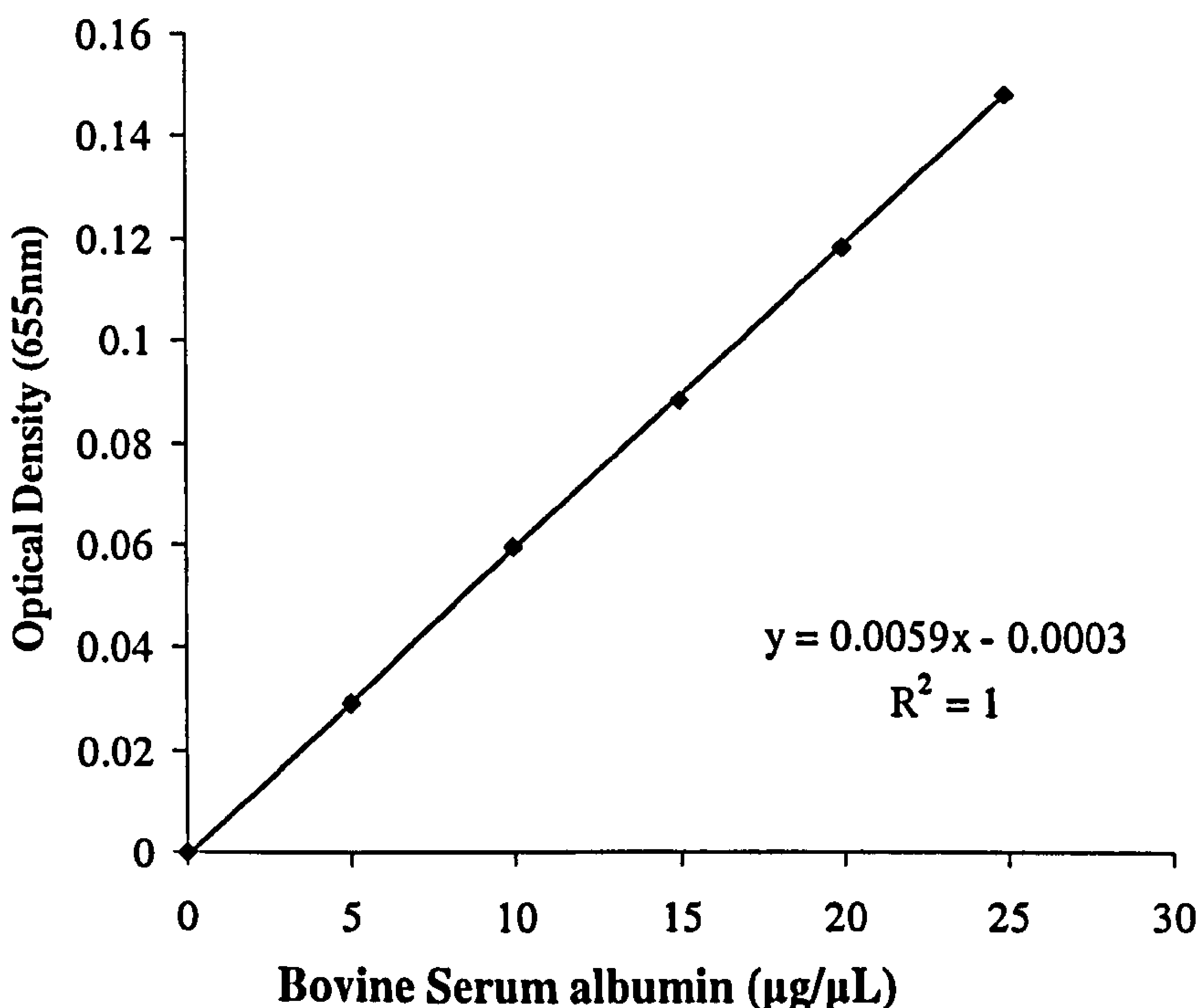

Figure AII.3.: The created standard curve is used as a reference to calculate protein content in samples of proteins extracted from cultured adipocytes, pre-adipocyte cells and tissue. Bovine serum albumin was diluted in mQH_2O and the absorbance is read at 655nm on a spectrophotometer.

AII.4. Glucose oxidase method

The glucose oxidase method is a technique that allows for accurate quantification of glucose concentration in both serum and plasma preparations of blood (Lott, 1975). Blood glucose is oxidized to gluconic acid, liberating hydrogen peroxide by an enzyme called glucose oxidase. The liberated hydrogen peroxide is converted by peroxidase to water and oxygen. The level of converted oxygen can be measured using an oxygen accepter termed 4 aminophenazone, which takes up oxygen forming a pink covered chromogen which can be detected.

AII.5. RNA extraction and Real time PCR

5.1. mRNA sequence

The deoxyribonucleic acid (DNA) or genetic code that encodes the protein structure is divided into introns (non-coding) and exons (coding) regions. When a gene is transcribed from the DNA strand by RNA polymerase, messenger ribonucleic acid (mRNA) is produced, which comprises only the exons. Each gene can comprise of a varied number of exons which describe the specific polypeptide chain to be assembled within the ribosomes located in the cytoplasm. Quantitative mRNA analysis determines the 'blueprint' prepared for protein synthesis.

5.2. RNA content in extracted samples

After extraction of RNA contaminating genomic DNA was removed using 1μl DNase diluted in 5μl reaction buffer. Protein contamination of RNA was calculated by measuring optical density of each sample at 260nm (OD_{260}) (wavelength for RNA and DNA detection) and 280nm (wavelength for protein detection). The ratio of these two readings is an indicator of the samples purity.

The conversion factor for RNA is 0.040μg/μl per OD_{260} unit and the quantity of RNA diluted is 4μl per 1ml. Therefore for a reading of OD_{260} 0.10; 0.10 * (1000/4) * 0.040 = 1 μg/mL.

5.3. RT-PCR

The polymerase chain reaction relies on complementary DNA as a template, therefore DNA must be synthesised from the extracted RNA. This process is termed reverse transcription (RTn), and allows the synthesis of complementary DNA (cDNA). This involves the 'reading' of the mRNA sequence and then a cDNA chain assembled that comprises the complimentary base to that on the mRNA strand (guanine always bonds with cytosine and adenine to thymine) (Figure AII.5.3.).

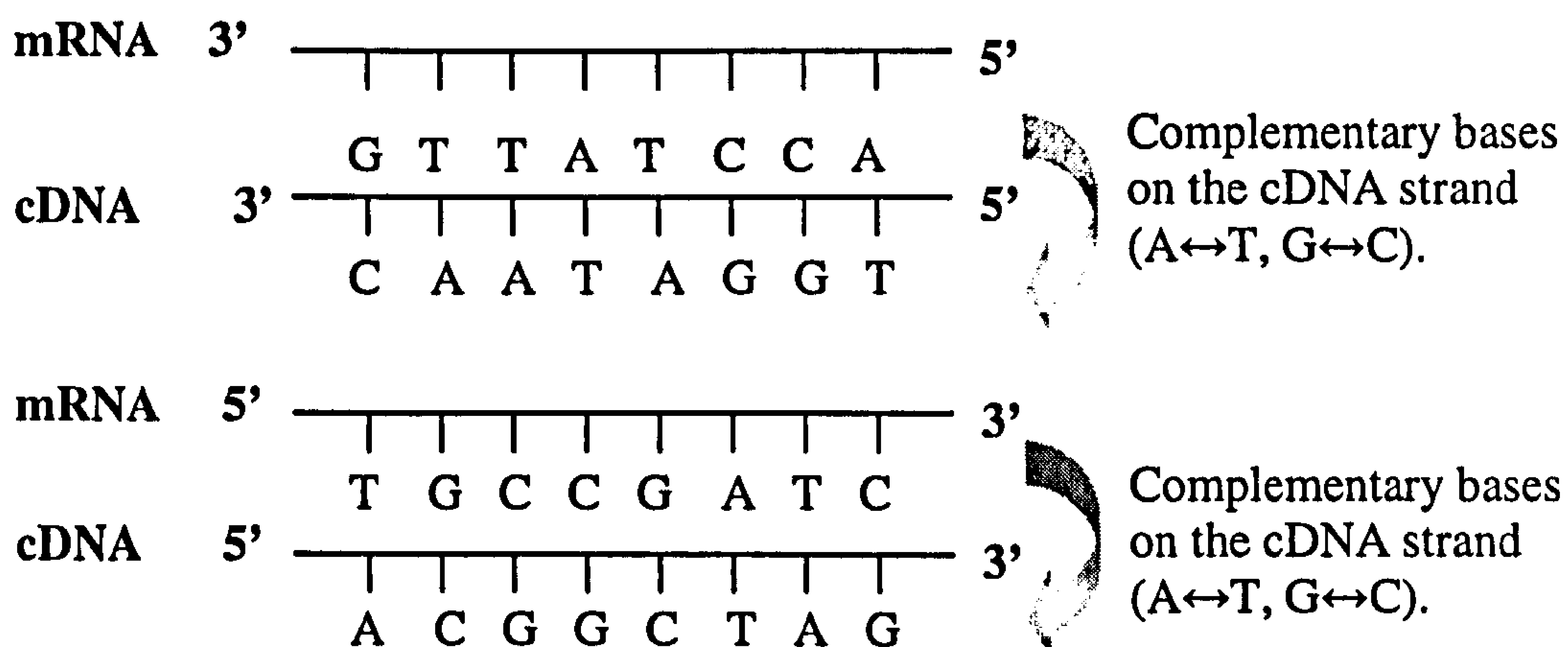

Figure AII.5.3.: This diagram illustrates the messenger RNA double strand, which is then digested in the reverse transcription process to yield single strands. Each cDNA strand is then composed using complementary bases to those on the mRNA strand.

5.4. Quantitative Real-Time PCR

Quantitative real-time PCR uses fluorescence technology to monitor amplification production. Real-Time PCR is designed to collect data as the reaction is proceeding, (*ie,* in real time). This is more accurate for DNA and RNA

quantitation and does not require laborious post PCR like the traditional PCR methods. The accumulation of product (amplicon) during the reaction is then measured at the exponential phase of the PCR reaction.

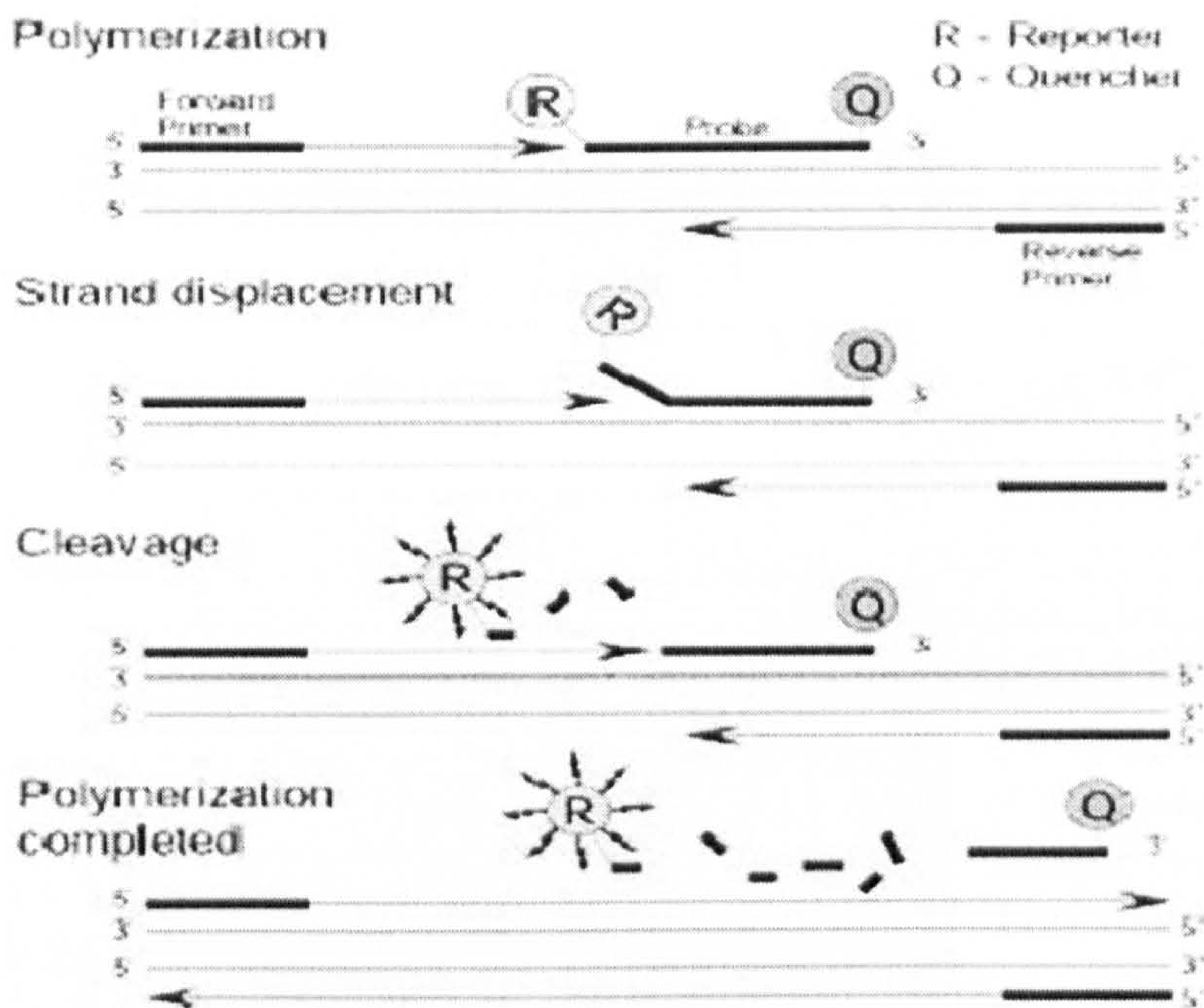

Figure AII.5.4.: Diagram showing TaqMan chemistry. When the probe is cleaved from the DNA during amplification the reporter dye is removed from the quencher and fluoresces.

In this study MRNA levels were analysed using an ABI 7700 Sequence Detection system which utilizes TaqMan chemistry. The TaqMan probe is designed with a high-energy dye termed a reporter at the 5' base end, and a quenching dye typically on the 3' base that suppresses the reporter. The quencher prevents emission of any fluorescence while the probe is intact. If the gene of interest is present, the probe anneals between the forward and reverse primer sites within the PCR product. When the Taq DNA polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe. This removes the reporter from the proximity of the quencher, resulting in a florescent signal that accumulates with each cycle. The fluorescent signal yielded can be quantitatively measured by a laser and charged coupled device (CCD) camera,

enabling real time detection of cDNA amplification. (Figure AII.5.4.).There is an increase in intensity with every amplification cycle. The threshold line is the level of detection at which a reaction reaches a fluorescent intensity above background (Figure AII.5.5.).

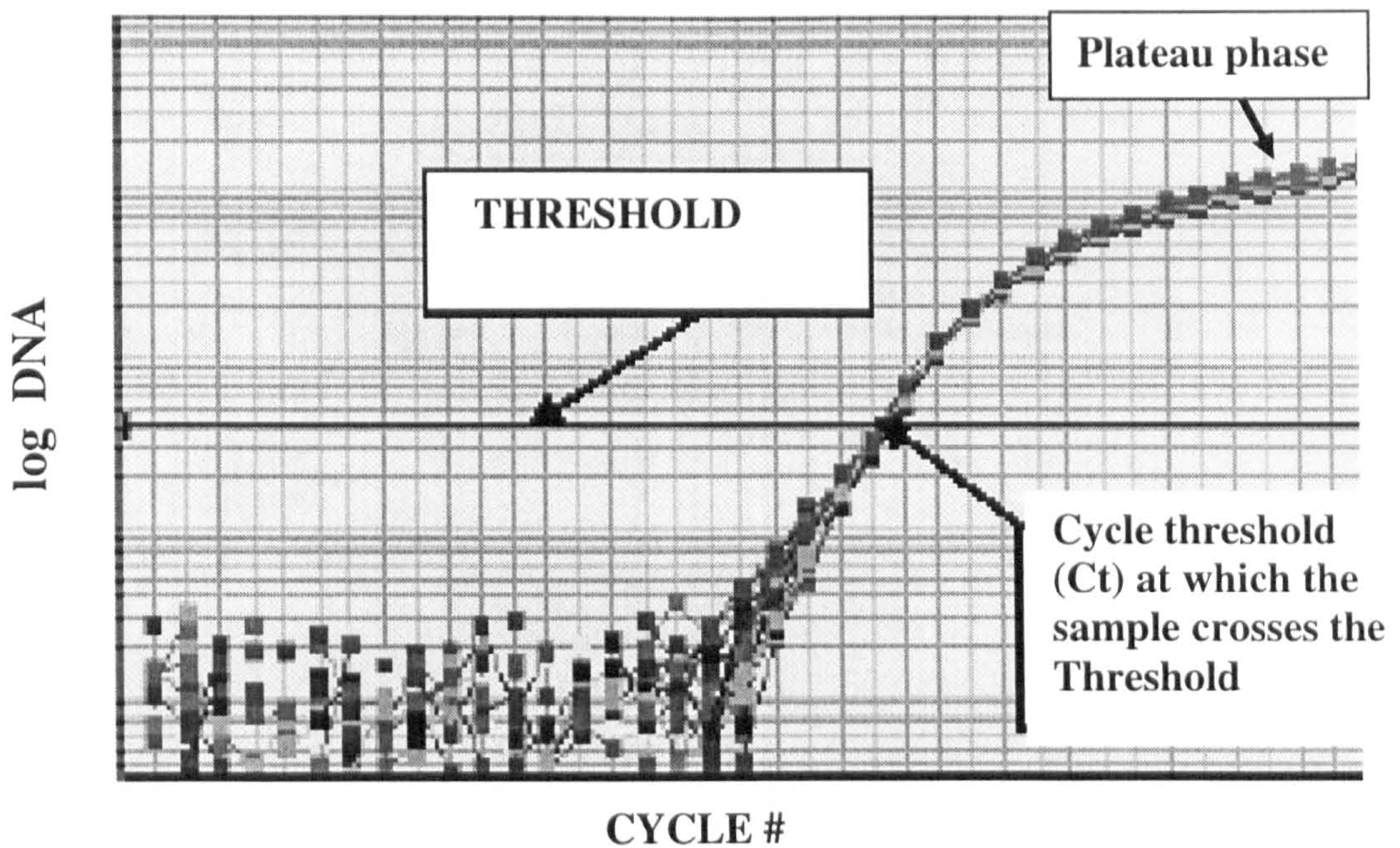

Figure AII.5.5.: Log view of real time PCR. The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the Cycle Threshold, Ct.

AII.6. Microarray

A schematic overview of the 'One-cycle target labelling' protocol is shown in figure II.6. and is derived from the 'GeneChip® Expression Analysis Data Analysis Fundamentals' manual and the 'GeneChip® Expression Analysis Technical Manual' both available at http://www.affymetrix.com.

A GeneChip® probe array consists of a number of *cells* (square-shaped areas on the array) and each contains many copies of a unique probe. Probes are tiled in probe pairs consisting of a perfect match (PM) and a mismatch (MM) which are only distinguishable by a base substitution in the middle of the MM probe

sequence. The significance of the differences between PM and MM was expressed by calculation of a *p*-value using a one-sided Wilcoxon's Signed Rank test.

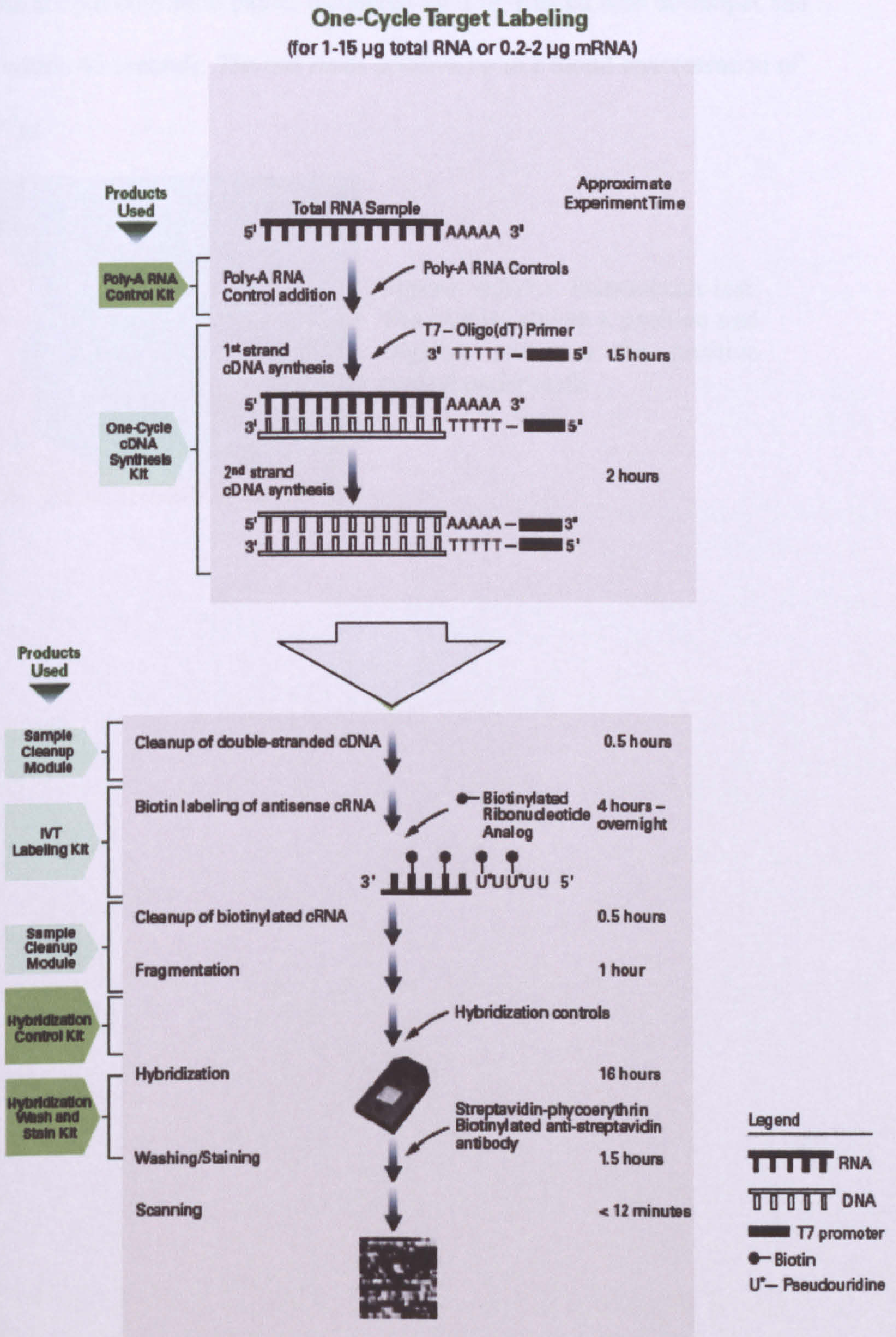

Figure AII. 6.: The 'One-cycle target labelling' protocol

AII.7. Haemoccult-test

The Haemoccult-test is used for screening of GI blood loss. A few drops of sample are put onto filter paper, incubated for 1 hr, treated with developer and read within 45 seconds. The test reads positive up to a blood concentration of $1 \times 10^{-4} \mu l$.

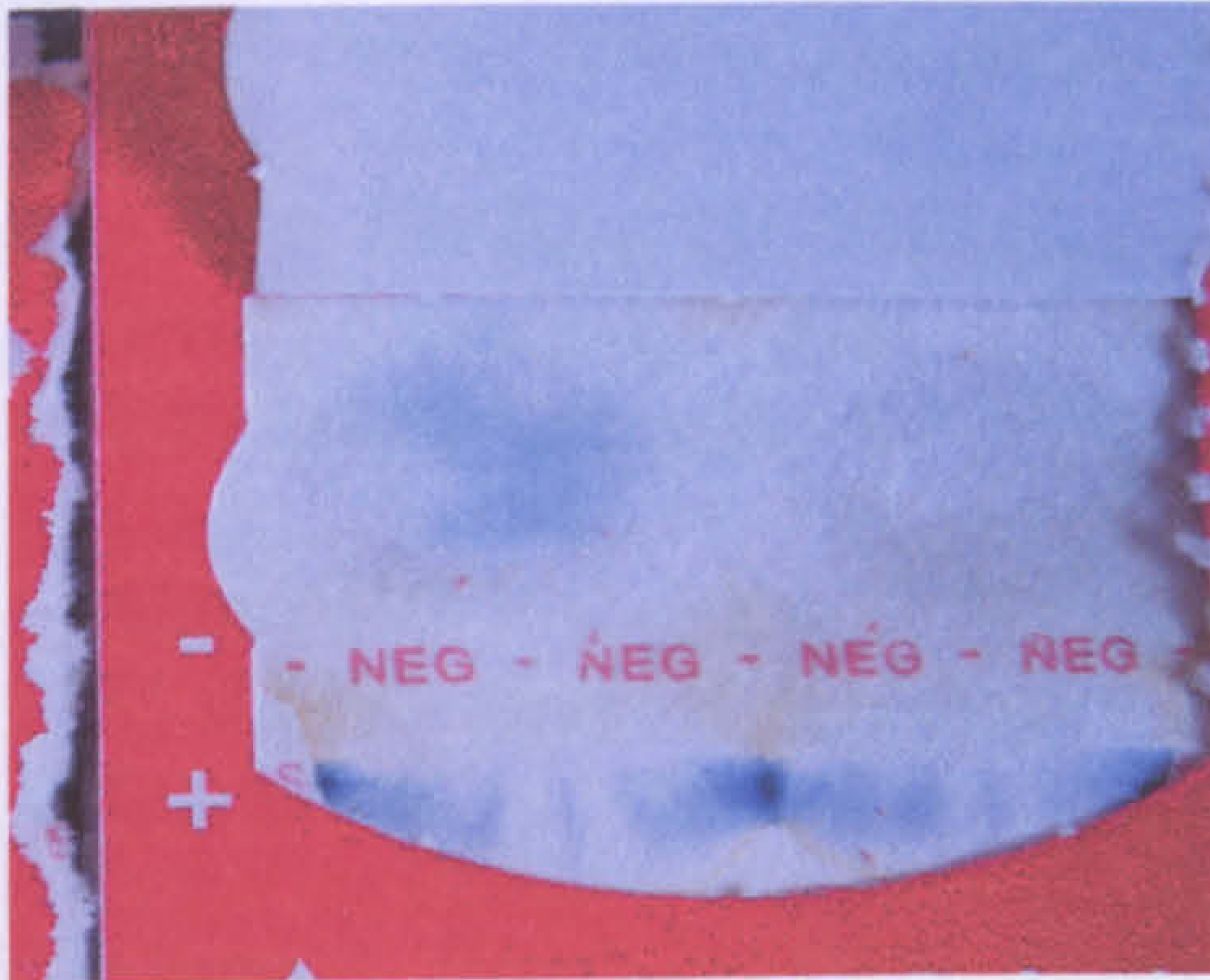

Figure AII.7.: Haemoccult-test. The picture shows a positive and negative test and the positive control underneath.

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