EFFECTS OF NEUTRALISING INTERLEUKIN-6 ON GLUCOCORTICOID-MEDIATED ADAPTATIONS TO STRESS IN RAT SKELETAL MUSCLE AND LIVER

Nathaniel W. Wilson

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Supervisor:

Prof. Kathryn H. Myburgh

Co-supervisor:

Dr. Ann Louw

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Declaration:

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for any degree.

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SUMMARY

This study (2 x 2 factor design) describes an investigation into the physiological interaction between the peripheral endocrine and cytokine systems after the organism has been exposed to psychological stress. An *in vivo* rodent model with two interventions was used: (1) mild psychological stress (immobilisation for 2 hours per day, for 4 days); (2) an anti-interleukin (IL)-6-antibody injection. Thirty-nine male Wistar rats were divided into 4 groups and given either the antibody (CA, control antibody) or stress (IP, immobilisation placebo), or both (IA, immobilisation antibody), or neither (CP, control placebo). Antibody and placebo (saline) were injected intraperitoneally. Differences between groups for the following parameters were determined in blood or metabolic tissues, viz. skeletal muscle and liver:

1) corticosterone concentrations,
2) glucocorticoid receptor (GR) binding capacity and
3) activities of metabolic enzymes, tyrosine aminotransferase (TAT) and glutamine synthetase (GS).

Groups IP and IA showed a significant loss in body mass (CP *vs.* IP, $p<0.01$; CA *vs.* IA, $p<0.001$), indicating a main effect of stress. The corticosterone concentrations of only group IP were significantly elevated compared to that of group CP (CP *vs.* IP, $p<0.01$), again indicating a main effect of stress. All three intervention groups (CA, IP, IA) had decreased GR binding capacity, with group IA showing a statistically greater decrease (CP *vs.* CA, $p<0.05$; IP *vs.* IA, $p<0.01$; CP *vs.* IP, $p<0.001$; CA *vs.* IA, $p<0.001$), indicating main effects of stress and antibody treatment. In groups IP and IA increased activities of both enzymes (TAT and GS) were measured (main effect of stress), with IA again showing the greatest statistically significant increase for both enzymes. The liver tissue displayed greater sensitivity to the stress and antibody regimes. This study provides the first conclusive *in vivo* evidence for IL-6 modulation of glucocorticoid action in peripheral tissues in response to mild psychological stress.

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OPSOMMING

Hierdie studie (met 'n 2 X 2 faktorontwerp) beskryf 'n ondersoek oor die fisiologiese interaksie tussen die perifere endokrien- en sitokiensisteme in organismes blootgestel aan psigologiese stres. Daar word gebruik gemaak van 'n *in vivo*-rotmodel met twee intervensies: (1) matige psigologiese stres (immobilisering vir 2 uur per dag vir 4 dae); (2) 'n anti-interleukin (IL)-6-antiliggaam inspuiting. Nege-en-dertig manlike Wistar rotte is in vier groepe verdeel en het òf antiliggaam (CA, antiliggaam kontrole), òf stres (IP, immobilisasie placebo), òf beide stres en antiliggaam (IA, immobilisasie antiliggaam) of geen behandeling ontvang (CP, placebo kontrole). Die antiliggaam- en placebo (soutoplossing)-inspuitings is intraperitoneaal toegedien. Verskille tussen die groepe van die volgende parameters, in metaboliese weefsels (skeletspier en lewer), was bepaal:

1) kortikosteroon konsentrasies,
2) glukokortikoïed reseptor (GR) bindingskapasiteit en
3) aktiwiteite van die metaboliese ensieme, tirosien aminotransferase (TAT) en glutamien sintetase (GS).

Groepe IP en IA het 'n beduidende afname in gewig getoon (CP *vs.* IP, $p<0.01$; CA *vs.* IA, $p<0.001$), wat 'n hoof-effek van stres aandui. Die kortikosteroon konsentrasies van slegs IP het beduidend toegeneem in vergelyking met CP (CP *vs.* IP, $p<0.01$), wat weereens 'n hoof-effek van stres aandui. Al drie intervensiegroepe (CA, IP, IA) het verlaagte GR bindingskapasiteit getoon, met IA wat 'n groot statistiese afname getoon het (CP *vs.* CA, $p<0.05$; IP *vs.* IA, $p<0.01$; CP *vs.* IP, $p<0.001$; CA *vs.* IA, $p<0.001$), wat hoof-effekte van beide stres en antiliggaam-behandeling aandui. In groepe IP and IA is toenames in beide ensiemaktiwiteitvlakke (TAT en GS ensieme) getoon (hoof-effek van stres), met IA wat weereens die grootste toename gewys het. Die lewer het ook verhoogde sensitiwiteit tot die stres- en antiliggaamregimente. Hierdie studie lewer die eerste daadwerklike *in vivo* bewyse vir IL-6 modulering van glukokortikoïedaksie in perifere weefsels na reaksie op psigologiese stres.

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ALPHABETICAL LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AIDS	acquired immune deficiency syndrome
AM	morning
AP-1	activating protein-1 (a transcription factor)
AVP	arginine vasopressin
B	corticosterone
BDNF	brain-derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
CBG	corticosteroid binding globulin
C/EBP	CCAAT/enhancer-binding protein
CNS	central nervous system
COX-2	cyclooxygenase-2
CREB	cAMP response element binding protein
CRF/CRH	corticotropin-releasing factor / corticotropin-releasing hormone
DBD	DNA binding domain
Dex	dexamethasone
DNA	deoxyribonucleic acid
DOC	deoxycorticosterone
E	epinephrine
et al.	and others
g	gram(s)
GC(s)	glucocorticoid(s)
GIF(s)	glucocorticoid-inducible factor(s)
Gln	Glutamine
Glu	Glutamate
GR(s)	glucocorticoid receptor(s)
GRE(s)	glucocorticoid response element(s)
GRU(s)	glucocorticoid response unit(s)
GS	glutamine synthetase (GS)
h	hour(s)
HNF-3	hepatic nuclear factor 3

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HPA	hypothalamic-pituitary-adrenal
HPPVs	hypothalamic-pituitary portal vessels
11-β-HSD	11-beta-hydroxysteroid dehydrogenase
hsp	heat shock protein
HTC	hepatic tissue culture
IL	interleukin (e.g. IL-1 and IL-6)
LBD	ligand binding domain
LC	locus ceruleus
LHPA	limbic- hypothalamic-pituitary-adrenal
LIFR	leukemia inhibitory factor receptor
LPS	lipopolysaccharide
ME	median eminence
min	minute(s)
MMTV-CAT	mouse mammary tumor virus – chloramphenicol acetyltransferase reporter plasmid
MPL	methylprednisolone
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NE	norepinephrine
NF-κB	nuclear factor kappa B
nGRE	negative glucocorticoid response element(s)
PM	afternoon
PNI	psychoneuroimmunology
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
SAM	sympathetic adrenal medullary axis
SERPINS	serine protease inhibitors and substrates
SNS	sympathetic nervous system
TAT	tyrosine aminotransferase
TNF	tumor necrosis factor
WBC(s)	white blood cell(s)

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To **Clothilde** and **Zeta**

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"…we have learned that the body possesses a complex machinery of checks and balances. These are remarkably effective in adjusting ourselves to virtually anything that can happen to us in life. But often this machinery does not work perfectly: sometimes our responses are too weak, so that they do not offer adequate protection; at other times they are too strong, so that we actually hurt ourselves by our own excessive reactions to stress."

Hans Selye (1956), 'The stress of life'

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CHAPTER 1 INTRODUCTION, HISTORICAL OVERVIEW AND AIMS

1.1 Introduction to stress and the adrenals

Hans Selye, once a medical doctor, is known as the "father of stress". Even though he never received a Nobel Prize for his ground-breaking achievements (despite being nominated several times), he single-handedly pioneered research on stress since the late 1930s. We owe the modern awareness of stress to him, as he first suggested that stress is a "state manifested by the specific syndrome which consists of all the non-specifically induced changes within a biologic system" (Selye, 1956). His research focused specifically on the physiological responses to stress.

Selye exposed rats to various types of stress for various durations before they were finally sacrificed and dissected. These dissections showed that stress causes 1) enlargement of the adrenal cortex, 2) atrophy of the thymus, spleen, lymph nodes and other lymphatic structures and 3) development of deep bleeding ulcers in the stomach and duodenum (Selye, 1956). These observations clearly indicated that 3 apparently separate systems were all affected by the common cause, "stress". Nevertheless, the adrenal gland and glucocorticoids (GCs) were the initial focus of stress research.

The experiments of Selye led to a classification of the progressive nature of stress effects on the body, particularly its influence on the adrenal glands. The "General Adaptation Syndrome" was described as having the following three stages:

- The 'Alarm Reaction' – characterized by surprise and anxiety, and generally considered to activate the 'fight or flight' reaction (Selye 1956).

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The adrenal glands secrete the hormones epinephrine, norepinephrine and hydrocortisone (cortisol) during this stage (Selye, 1956).

- The 'Resistance Phase' – with continued exposure to stress, the body prepares for future stress and adapts its response to the stressor(s). This phase is characterized by adrenal hypertrophy as well as a more rapid return to the resting state after an acute stressor (Selye, 1956).
- The 'Exhaustion Phase' – the adrenal glands can no longer meet the demands placed on them due to prolonged stress (Selye, 1956).

This endocrine research laid the basis for subsequent biochemical, neurophysiological and, more recently, psychoneuroimmunological (PNI) stress research. PNI is considered a fairly new concept (Benowitz, 1996), but is itself based on a long history of integrative research on the GCs and the immune system.

Due to our multidisciplinary biochemical and physiological interests, we sought to investigate the stress-sensitive interaction between GCs and the cytokine system in an *in vivo* rat model.

1.2 Glucocorticoids and the immune system

Although Selye was one of the most prolific researchers of GC physiology, he turned out to be wrong about some critical aspects (Sapolsky *et al.,* 2000). He claimed that GC excess could cause arthritis, allergies and collagen-related disorders (Selye, 1956). However, potent anti-inflammatory GC actions were discovered which did not fit into the paradigm that GCs cause illnesses (Hench *et al.,* 1949; reviewed by Munck *et al.,* 1984). Rather, GCs play a role in the regulation of the immune system. The following paragraphs will briefly outline the historical development of our current concept that not only do GCs communicate with the immune system, but the immune system also communicates with the endocrine stress axis.

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Actually, it was as early as 1855 that Thomas Addison observed that a patient with adrenal insufficiency had an excess of circulating lymphocytes (Addison, 1855). In 1924, Jaffe removed the adrenal glands ('suprarenalectomy') and observed a subsequent hypertrophy of the thymus (Jaffe, 1924). The thymus is the organ responsible for manufacturing mature lymphocytes (Vander, 2001). It is with this background that Selye performed his famous studies published in 1925. Following his results, it was realized that no matter what the nature of the injury or stressful insult, the laboratory animals developed enlarged adrenal glands and an involuted thymus (Selye, 1956). These early studies clearly suggested a close relationship between adrenal gland physiology and immune capacity.

The first direct proof that adrenal GC secretion plays a significant role in regulating immune processes was provided between 1936 and 1949 by the Nobel Prize winners, Kendall, Hench and Reichstein. They isolated the active component secreted by the adrenal cortex (cortisone) and demonstrated its ability to suppress inflammation (Hench *et al.*, 1949). However, it was only in the 1980's that Munck and Guyre interpreted this relationship more clearly: the increased secretion of GCs during stress is important in preventing the inflammatory and immune response systems from over-reacting (Munck and Guyre, 1986; Munck *et al.,* 1984). Munck *et al.* proposed in their 1984-review that "(a) the physiological function of stress-induced increases in GC levels is to protect not against the source of stress itself, but against the normal defense reactions that are activated by stress; and (b) the GCs accomplish this function by turning off those defense reactions, thus preventing them from overshooting and themselves threatening homeostasis". Although these contributions of Munck *et al.* are regarded as very significant, the efforts of Marius Tausk in 1951 were not recognized. According to Sapolsky *et al.*, Tausk published a similar idea to that of Munck and his colleagues in a pharmaceutical company's periodical (Sapolsky *et al.,* 2000). Tausk illustrated his idea by comparing stress to a fire and the role of GCs to that of preventing water damage rather than putting out the fire (Tausk, 1951). In 1989, a subsequent study was able to link continuous

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immune inflammatory responses (in streptococcal cell wall arthritis-susceptible Lewis rats) more definitively to inadequate counter-regulation of GC output (Sternberg *et al.,* 1989). Therefore, although GCs are indirectly involved (notably by the absence of its main actions), they do not cause immune diseases as suggested almost 60 years before by Selye.

Studies showing immunosuppression by GCs resulted in widespread use of GC-based therapies for autoimmune and inflammatory diseases (for a recent review see Turnbull and Rivier, 1999). However, it was the pioneering work of Besedovsky, del Rey, Sorkin and colleagues that provided the real mechanisms for the physiological role of GCs in preventing overactivity of immune reactions (Besedovsky *et al.* 1979; Del Rey *et al.* 1984). Their contributions actually laid the foundation for Munck's review (Munck *et al.* 1984). These researchers showed that the influence of GCs on immune processes is much more complex than a generalized suppression of immune activity. Besedovsky *et al.* suggested that immune activity itself also influences GC secretion (Besedovsky *et al.,* 1975). This and other studies suggested the existence of an immune-neuroendocrine regulatory feedback mechanism in which immune cells indirectly limit their own activity by secreting molecules that stimulate the secretion of adrenal GCs (Besedovsky *et al.,* 1975; Besedovsky *et al.,* 1981). This concept is now called the 'bi-directional communication' between the immune and endocrine systems - Blalock and colleagues were the first to describe molecules actually responsible for such bi-directional communication (Blalock *et al.* 1985, Blalock, 1989; Weigent & Blalock, 1987).

1.3 Glucocorticoid-inducible factors

Several studies identified factors released under physiological stress that also seemed to be capable of communicating with the HPA axis. For example, Besedovsky *et al.* showed in 1985 that stimulated lymphocytes produce a "glucocorticoid increasing factor" (GIF) that increases plasma ACTH and

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corticosterone levels in rats and that hypophysectomy prevented this response (Besedovsky *et al.* 1985). This GIF was later identified as interleukin-1 (IL-1) (a cytokine – see Chapter 2, section 2.5). GIF which was shown to cause fever via signalling of the central nervous system (CNS) after systemic invasion by pathogens (for reviews see Atkins, 1960; Kluger, 1991). IL-1 plays an important endogenous role in regulating the HPA axis during viral disease (Silvermann *et al.,* 2005). Also, it was realized that 'extrahypothalamic' corticotropin-releasing factors, released by injured tissue, were also cytokines (Lymangrover and Brodish, 1973; Brodish, 1977a; Brodish, 1977b).

The monokines, IL-1 and interleukin-6 (IL-6, also known as hepatocyte-stimulating factor), stimulate adrenocorticotrophic hormone (ACTH) secretion (Woloski *et al.* 1985). (The monokines are part of the cytokine family – see Chapter 2, section 2.5, for further explanation). Definitive evidence was provided by Besedovsky *et al.,* who demonstrated that, (a) in normal mice, systemic administration of monocyte-derived or recombinant IL-1 increases plasma ACTH and GC concentrations; and (b) in rats, neutralization of endogenous IL-1 inhibits the GC response to experimental viral infection (Besedovsky *et al.* 1986). These and other studies provided evidence that the immunoregulatory cytokines are a key, centrally active link between the immune and neuroendocrine systems (Besedovsky *et al.* 1986; Healy *et al.* 1983; McGillis *et al.* 1985; Woloski *et al.* 1985).

Further studies implicated the hypothalamus as the particular central site at which the HPA axis response to IL-1 is mediated. The ability to increase the secretory activity of the HPA axis is a fundamental biological property of several other cytokines as well: various interleukins, tumor necrosis factors, chemokines, hematopoietins, interferons, growth and neurotrophic factors (Turnbull and Rivier, 1999). In this respect, it is also important to note that cytokines stimulate the secretion of other cytokines, e.g. IL-1 potently stimulates the secretion of IL-6 (Libert *et al.,* 1990; Nietfeld *et al.,* 1990; Shalaby *et al.,* 1989; Tosato and Jones,

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1990). Ruzek *et al.* suggested that IL-6 is actually the circulating mediator of IL-1 actions (Ruzek *et al.,* 1997). Hence, recent and current research is focusing more and more on the role of IL-6 (e.g. Vanden Berghe *et al.,* 2000; Samuelsson *et al.,* 2004).

Cytokine up-regulation of the hypothalamic-pituitary-adrenal (HPA) axis may occur not only during infection, inflammation and trauma, but also during periods of psychological stress unrelated to the presence of disease or tissue damage (Turnbull and Rivier, 1999). The role of IL-6 in the adrenal and target tissue response to psychological stress is the focus of this thesis.

1.4 Corticosteroid receptors

The mobilisation of GCs, whether through direct activation of the HPA-axis during stress or through indirect activation by cytokines, acutely affects a large variety of tissues. A popular approach to studying the molecular mechanisms whereby GCs exert their effects, is to study the GC receptors (GRs) (Arriza *et al.,* 1987; Evans, 1988; Funder *et al.,* 1988; Whorwood *et al.,* 1992; Rupprecht *et al.,* 1993; Trapp *et al.,* 1994; Listwak *et al.,* 1996; Korn *et al.,* 1998; Yudt and Cidlowski, 2002; Almawi & Melemedjian, 2002; Schäcke *et al.,* 2004). Two types of GRs exist, the Type 1 (mineralocorticoid receptor, MR) and Type 2 (the classical glucocorticoid receptor, GR) (Listwak *et al.,* 1996).

The actions of corticosteroids (e.g. aldosterone, corticosterone and cortisol) are mediated by both MRs and GRs, which function as hormone-activated transcription factors that regulate target gene expression (Evans, 1988). In the CNS the MRs and GRs mediate both the feedback inhibition of HPA axis-activity by cortisol or corticosterone as well as feedforward functions such as regulation of circadian rhythms (Martin & Reichlin, 1987; De Kloet, 1991). There is a higher affinity between cortisol and MR than between cortisol and GR (Listwak *et al.,* 1996), therefore occupancy of the MR in the hippocampus occurs with lower

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levels of cortisol (associated with non-stress conditions or general homeostasis). This appears to set the activation level of the HPA axis, whereas occupancy of the GR during stress-associated conditions with higher levels of cortisol results in feedback inhibition of the HPA axis (Listwak *et al.,* 1996). The importance hereof lies in the realization that the MR and GR have different roles, although both via cortisol, in regulation of the HPA axis, which results in a coordinated control of homeostasis for this axis (De Kloet, 1991).

Peripherally, the MR is expressed in high concentrations in the kidneys and large intestine, where it mediates the regulation of water and electrolyte balance by aldosterone (Listwak *et al.,* 1996). GRs are constitutively expressed in most tissues throughout the body, but GR density varies under different physiological conditions (Okret *et al.,* 1991). Selectivity of both types of receptors for either cortisol or aldosterone is controlled by the expression of 11-β-hydroxysteroid dehydrogenase (11-β-HSD), an enzyme that exists in two isoforms, 11-β-HSD-type 1 and 2 (Arnold *et al.,* 2003). The former isoform is primarily a reductase, *in vivo,* and forms active cortisol from inactive cortisone, whereas the latter is an oxidase that catalyzes the inactivation of cortisol and therbey renders MR-specificity for aldosterone (Baker, 2003b; Arnold *et al.,* 2003). It follows that differential expression of the 11-β-HSD isoforms determines the specific tissue responses to either aldosterone or cortisol (or corticosterone), via the MR or the GR.

The major interest of this thesis pertains to the peripheral activity of GR after psychological stress. Cortisol (humans) or corticosterone (rats) is present in the circulation in much higher concentrations than aldosterone (Baker, 2003a) and the scope of this thesis is to investigate the peripheral activity of GR in major metabolic tissues such as liver and muscle. Also, 11-β-HSD-type 1 activity has been found in the liver, which indicates a more specific role for GR in the liver (De Kloet *et al.,* 1998).

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Further interest in the GR is heightened by the ongoing controversy regarding whether or not GR expression is increased or decreased after increases in the GC concentrations during stressful situations. Many researchers have found that an increase in GCs lead to down-regulation of GRs during exposure to stress (for a summary, see discussion of Al-Mohaisen *et al*, 2000), possibly providing a mechanism whereby the effects of prolonged GC action is limited. Other researchers, in contrast, found increases in GR number and activity (Al-Mohaisen *et al.*, 2000; Sun *et al.,* 2002). This discrepancy is discussed in Chapter 5.

The function of the GR is to bind to GC response elements (GRE) in the promoters of target genes, thereby regulating the expression of many enzymes and proteins (see examples in section 1.5 and review by Newton, 2000). As a primary feature, GCs, via the GR, induce gluconeogenesis in the liver as a means to increase the availability of glucose for energy production during stress (Trenkle, 1981; Sun *et al.*, 1998). GCs, via GR, also induce mobilisation of substrates for gluconeogenesis from e.g. muscle tissue (Salway, 1994). In this respect, our interest is focused on the activity of GR in the cytosol of the metabolic target tissues, muscle and liver. Cytosolic GR translocates to the nucleus and regulates the expression of genes (for a detailed discussion on these mechanisms, see Chapter 2). Examples of GC-inducible enzymes are discussed below.

1.5 Glucocorticoid-inducible enzymes

Glucocorticoids induce gluconeogenesis via the transcriptional up-regulation of specific enzymes involved in amino acid metabolism in various metabolic tissues. Our interest in the peripheral effects of glucocorticoids was investigated by focusing on the activities of two well-known GC-inducible metabolic enzymes, viz. glutamine synthetase (GS) and tyrosine aminotransferase (TAT).

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The genes that encode for these enzymes are rendered GC-inducible by the existence of GREs in their promoter regions. It is a well-known fact that TAT and GS activities increase during stress (Al-Mohaisen *et al.,* 2000; Vats *et al.,* 1999). The metabolic purpose of such up-regulation lies in the subsequent alteration of amino acid metabolism, which ultimately serves to supply amino acids that are used in energy production by other organs, especially the liver (Salway, 1994). In addition, GS and TAT represent two of the most well-known GC-inducible enzymes. Despite this, no evidence exists which proves a relationship between their activities, especially after exposure to psychological stress. Our focus, therefore, is to separately investigate the activities of these enzymes during psychological stress and to subsequently determine whether there is any correlation between TAT and GS function.

1.6 Classification of GC function

In accordance with a recent classification (Sapolsky *et al.,* 2000), one can distinguish between two classes of GC actions: *modulating actions* (actual GC actions resulting in the effects that alter the organism's responses to the stressor and include the permissive, suppressive and stimulating GC actions) and *preparative actions* (actions that do not affect the immediate response to a stressor but modulate the organism's response to a subsequent stressor i.e. the initial stress response results in an adaptation possibly at the level of gene expression). The preparative actions of this classification correlate with Selye's 'resistance phase' of the general adaptation syndrome (section 1.1). In essence, the body adapts to the stressor and prepares for future stressful encounters.

Brief explanations of modulating GC actions are (Sapolsky *et al.,* 2000):

- Permissive GC actions are exerted by GCs present *before* the stressor and therefore prime the defense mechanisms by which an organism responds to stress. I interpret this to mean that a certain range of corticosterone/cortisol concentrations is maintained in circulation

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('baseline' levels) to ensure a certain amount of activated receptors in tissues for baseline modulation of gene activity.

- Suppressive GC actions are attributable to the stress-induced rise in GC levels. These actions have an onset from about an hour or more after the onset of the stressor. These actions represent stress-activated defense reactions and prevent the stress system from overshooting.
- Stimulating GC actions are also attributable to the stress-induced rise in GC levels. These actions, too, have an onset from about an hour or more after the onset of the stressor. They are the reverse of the above-described suppressive actions because they enhance the effects of the first wave of hormonal responses to stress (i.e. the catecholamines, see Chapter 2). Permissive and stimulating GC actions are collectively referred to as helping to mediate the stress response.

Whether or not a target tissue (or protein) responds suppressively or stimulatory is not well understood, which provides impetus for our current research interests.

1.7 Current issues

Tracing the historical development of our understanding of stress, cytokines, GCs and GRs has highlighted, in particular, our improved understanding of their roles in the HPA-axis response but not the subsequent tissue responses. The multiple mechanisms whereby corticosteroids and cytokines exert their effects on the body of the mammalian organism are still not completely understood, hence the importance of establishing these mechanisms more clearly. The close bidirectional communication between the endocrine and cytokine systems has previously mainly been studied in models of infection or fever (e.g. Busbridge *et al.,* 1990; Dunn, 1993; Dunn and Vickers, 1994; Ruzek *et al.,* 1997). Very few *in vivo* studies have investigated the effect of psychological stress on cytokines in conjunction with endocrine parameters, particularly not with inclusion of receptor analyses at the level of target tissues other than the CNS.

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Recent evidence suggests that many cells other than immune cells also synthesize and are responsive to cytokines. This is attributed to interaction at a molecular level between the activated GR and transcription factors or enzymes (e.g. nuclear factor (NF)-κB and cyclooxygenase (COX-2)) that modulate the expression of cytokines and other immune parameters (Rivest, 2001).

Recently, blocking the effects of one or another cytokine in the cascade of the inflammatory response has become possible by using various models, e.g. knockout mice models (e.g. Xing *et al.,* 1998), or administration of antiserum or antibody, e.g. antibodies directed against IL-6 (Arruda *et al.,* 2000; Marby *et al.,* 2001). Very few studies using blocking models have assessed GR responses. Such studies can illuminate our understanding of the complex response to stress or illness.

1.8 Hypothesis and aims

During psychological stress, IL-1α and –β are the main cytokines released from the hypothalamus. As a result, IL-6 release into circulation (from e.g. immune cells) is stimulated, which is responsible for a host of peripheral effects. Specifically, we hypothesize (based on previous *in vitro* work) that IL-6 is responsible for modulation of GC action in circulation as well as in peripheral tissue, and that this IL-6 modulation of GC action is essential in preventing the stress-induced GC response from exerting deleterious metabolic effects.

Thus, the aim of this study was to investigate the effects of blocking IL-6, *in vivo,* during psychological stress on:

- two parameters of stress, namely, body mass and circulating corticosterone levels;
- GR activity levels by measuring GR binding capacity in the liver and muscle;

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- the activities of two GC-inducible enzymes, GS and TAT, in liver and muscle.

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CHAPTER 2 LITERATURE REVIEW

2.1 The stress response

2.1.1 Stressors

A stressor has been defined as any intrinsic or extrinsic threat or challenge to the homeostasis of an organism (Chrousos & Gold, 1992). Thus, stress itself can be defined as a state of disrupted homeostasis to which the organism adapts by employing a host of physical or mental reactions (adaptive responses) that attempt to counteract the effects of the stressors in order to reestablish homeostasis (Chrousos & Gold, 1992). The adaptive responses are further divided into general (non-specific) and specific responses (Chrousos & Gold, 1992). Typical stressors include conditions such as, for example, hunger or fasting, viral or bacterial infections, inflammation, exercise, emotional and psychological stress. The response to stress is typically characterised by surprise, anxiety and alarm (Selye, 1956).

Stressful experiences can either be acute or chronic. Acute stressors are perceived as intensely stressful experiences that may last for a few seconds to a few hours. In contrast, chronic stressors are stressful experiences that persist for a few days or even months and may be less intense. Due to the increase in the prevalence of many long-term diseases, the focus of the last two decades of research shifted from the study of acute effects to the study of the effects of long-term chronic stressors. The current understanding, as explained by Tsigos and Chrousos, is that chronic activation of the stress system is likely to lead to diseases already described by Selye in 1936 (Tsigos and Chrousos, 2002; Selye, 1936).

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In attempts to study and clarify the mechanisms underlying stress responses, researchers have used many different models of stressors, including caloric restriction, tissue injury, sepsis, electroshock, endotoxin treatment, exposure to novel environments, conditioned aversive stimuli, physical restraint and immobilisation stress (Zhou *et al.,* 1993; Gursoy *et al.,* 2001; Turnbull & Rivier, 1999). Some models are severe, whereas others are mild. In addition, the duration that stress is applied in the various models, or even in the same model, may differ substantially. This, in part, depends on whether the interests of the researchers are in the physiological or psychological responses to stress. Other factors that influence the chosen duration of stress exposure may include whether or not the researchers are interested in the early adaptive response or effects of long-term exposure. Immobilisation in rats is known as an inducer of psychological stress and downstream physiological adaptations have been documented, hence the use of this model for the purpose of this study (Al-Mohaisen *et al.,* 2000; Gursoy *et al.,* 2001). The term "chronic immobilisation stress" has been used to describe exposure lasting as briefly as e.g. 7 days (Aguilera *et al.,* 1996) and as long as e.g. 2 months (Hu *et al.,* 2000). Although the rationale is similar, the model we employ differs in severity from traditional methods of restraint stress (for details see Reinhardt *et al.,* 1995) and could therefore be considered as a model of milder stress.

In the following sub-sections, I will review the stress response: firstly, regarding its function; and finally, by discussing the two main pathways of response.

2.1.1.1 Function of the stress response

As an essential tool for survival, the stress response functions to re-establish and preserve homeostasis by activating central and peripheral responses for adaptive purposes (Chrousos & Gold, 1992). During the stress response, the central neural pathways (see section 2.1.1.2) mediate functions such as arousal, vigilance, alertness, cognition, focused attention and appropriate aggression;

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with simultaneous inhibition of feeding and reproductive urges (Chrousos & Gold, 1992). The acute peripheral changes include a shift in metabolism and redirection of oxygen and nutrients to the central nervous system and the stressed body site(s) (Chrousos & Gold, 1992). The peripheral changes include increases in cardiovascular tone (that lead to elevations in blood pressure and heart rate), increases in respiratory rate, gluconeogenesis and lipolysis thus promoting enhanced availability of vital substrates (Chrousos & Gold, 1992). The adaptive responses include a peripherally mediated restraint of growth and reproduction, both of which preserve energy, so that available energy can be used more efficiently in other areas of the body.

One major effect of the stress response is the release of glucocorticoids (the classical 'stress hormones') from the adrenal glands, as mediated through activation of the HPA axis (sections 2.1.1.2 and 2.2). As mentioned in Chapter 1, Tausk compared stress to a fire and the role of glucocorticoids to that of preventing water damage rather than putting out the fire (Tausk, 1951). Munck *et al.,* in their frequently quoted review, provided a more eloquent explanation for the role of glucocorticoids in stress: (a) the physiological function of stress-induced increases in glucocorticoid levels is not to deal with the source of stress itself, but to protect against the normal defense reactions that are activated by stress; and (b) the glucocorticoids accomplish this function by turning off those defense reactions, thus preventing them from overshooting and themselves threatening homeostasis (Munck *et al.,* 1984).

2.1.1.2 Main pathways of the stress response

The stress response is initiated and maintained by both central and peripheral components. Although the limbic-hypothalamic-pituitary-adrenal (LHPA) axis is the endocrine system most closely linked to stress in mammalians, the role the limbic system (especially the hippocampus) plays is not well understood yet (Vazquez, 1997). Of note is the fact that hippocampal GR expression is influenced by early environmental events in rats, which in turn may affect HPA

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axis function (Mohammed & Henriksson, 1993). Within the hypothalamus, the paraventricular nucleus (PVN) represent the final common integration of the stress response in the brain and it contains neurons for both corticotropin-releasing hormone (CRH, also known as CRF, corticotropin-releasing factor) and arginine-vasopressin (AVP). Thus, for our purpose, the hypothalamus represents the top controlling structure of the HPA axis. The brain stem contains the locus ceruleus (LC)-norepinephrine system (LC/NE system), which is a collective term for the locus ceruleus and other noradrenergic cell groups of the medulla and pons (Tsigos & Chrousos, 2002). Together, the PVN (hypothalamus) and LC (brain stem) constitute the central control systems of the stress response. The peripheral component of the stress response is mediated by the HPA axis and the efferent sympathetic/adrenomedullary system (Tsigos & Chrousos, 2002). The HPA axis mediates the release of cortisol or corticosterone whereas the LC/NE system mediates the release of NE, E and IL-6 (Tsigos & Chrousos, 2002). A simplified schematic representation of the central and peripheral components of the stress response is shown in Fig. 2.1.

2.1.2 The typical vertebrate endocrine stress response

When faced with a stressor, the vertebrate organism responds by initiating an endocrine stress response that primarily consists of two waves of hormonal secretion (Sapolsky et al., 2000). The first wave occurs within seconds after exposure to the stressor and involves enhanced secretion of catecholamines (NE and E) from the sympathetic nervous system as well as hypothalamic release of CRH into the hypothalamic-pituitary portal circulation, which is followed by enhanced secretion of ACTH (Sapolsky et al., 2000). The hormones of the first wave exert most of their effects via rapid second messenger cascades in target tissues within seconds and up to a few minutes (Sapolsky et al., 2000).

About four times more E is secreted than NE in humans and these hormones exert similar functions to those of sympathetic nerves, which include:

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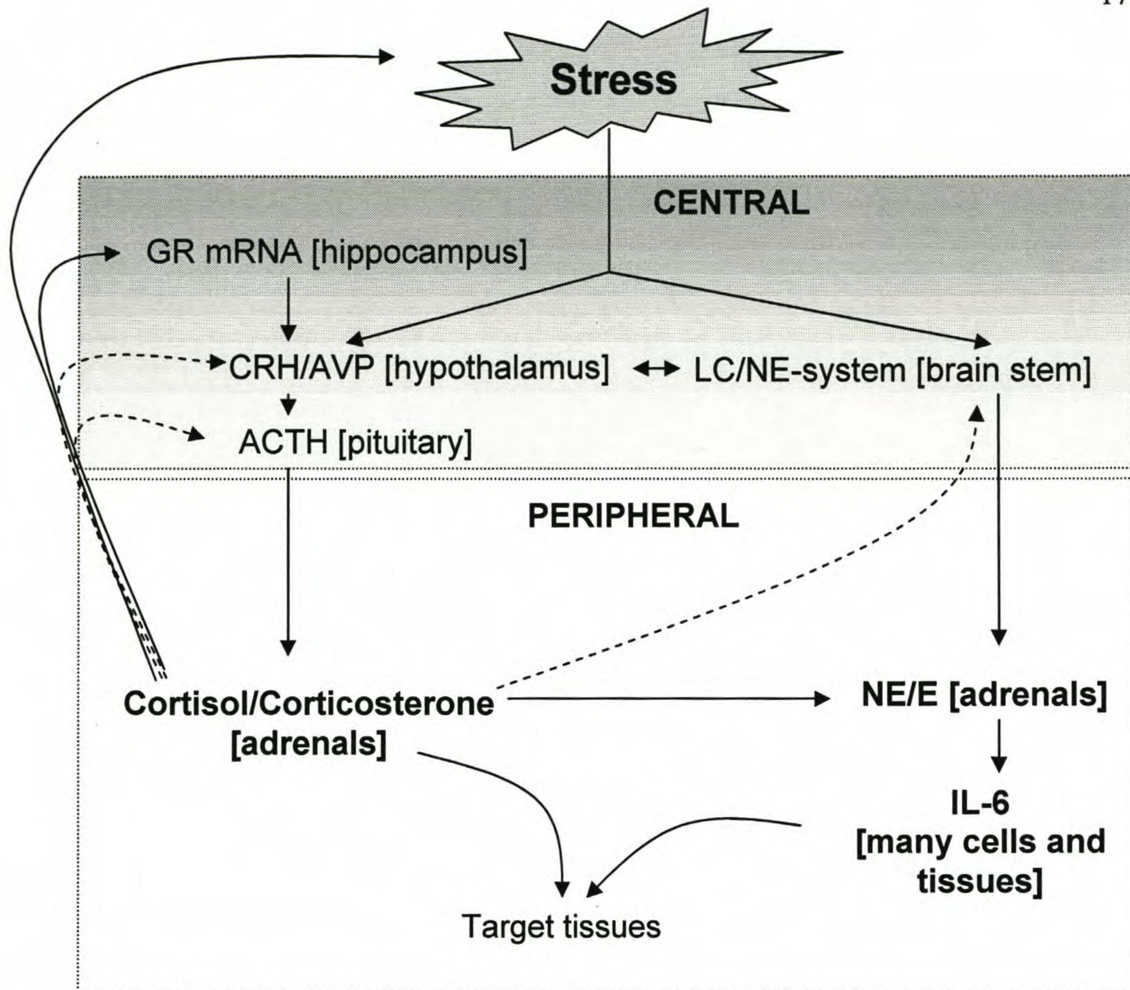

i. increased hepatic glycogenolysis and muscle glycolysis;

Figure 2.1 The two main pathways of the stress response. In mammals, the stress response is mediated by the HPA axis and the LC/NE system. The key peripheral effectors of these two systems are cortisol (humans) or corticosterone (rats and mice) and IL-6, respectively (adapted from Tsigos & Chrousos, 2002). Abbreviations: ACTH, adrenocorticotropin-releasing hormone; AVP, arginine-vasopressin neurons; CRH, corticotrophin-releasing hormone neurons; IL-6, interleukin-6; LC, locus ceruleus; NE, norepinephrine. Solid lines indicate stimulatory effects; dashed lines indicate inhibitory effects.

ii. increased catabolism (breakdown) of adipose tissue thus supplying glycerol for gluconeogenesis and fatty acids for oxidation;

iii. decreased fatigue of skeletal muscle;

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iv. increased cardiac output secondary to increased cardiac contractility and heart rate;
v. vasoconstriction in viscera and vasodilation in skeletal muscle, thus improving muscle function and usefulness;
vi. increased ventilation and
vii. increased coagulability of blood (Vander, 2001).

In contrast, the second wave is slower than the first and involves the steroid hormones. During this time, GC secretion is stimulated and gonadal steroid secretion declines (Sapolsky *et al.,* 2000). The bulk of steroid actions are genomic, therefore very few GC actions are exerted until about an hour after the onset of the stressor (Sapolsky *et al.,* 2000). GCs cause numerous metabolic effects such as increased blood glucose levels, stimulation of gluconeogenesis in the liver and the mobilisation of both amino and fatty acids from muscle and adipose tissue, respectively (Reichardt & Schutz, 1998).

2.2 Glucocorticoid physiology

Upon ACTH stimulation, GCs are synthesized in the adrenals (will be discussed in section 2.2.1) and secreted into the blood. GCs are then transported (will be discussed in section 2.2.2) to target organs. The molecular mechanisms of GC action will be discussed in section 2.2.3. Prolonged chronic stress with attendant elevated GC levels may result in GC resistance and this section will be concluded with a discussion of this phenomenon (section 2.2.4). Two GC-inducible genes affecting metabolism of amino acids in response to stress will be highlighted namely TAT (section 2.3.1) and GS (section 2.3.2). For a better understanding of the role of GCs, the following sections will take a closer look at basic GC physiology.

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2.2.1 Glucocorticoid synthesis

As mentioned earlier (section 2.1.1.2), the PVN, situated in the hypothalamus, is the primary CNS nucleus involved in the regulation of the pituitary-adrenal axis by being the principle CNS source of CRF – the major regulator of pituitary ACTH secretion (Rivier and Plotsky, 1986).

The median eminence (ME) is the base of the hypothalamus and contains capillaries that combine to form the hypothalamic-pituitary portal vessels (HPPVs) that pass down the pituitary stalk into the pituitary gland (Vander, 2001). These vessels represent an unusual blood-vessel connection between the hypothalamus and pituitary, and thereby offer a local route for blood flow directly from the hypothalamus to the anterior pituitary. The CRF hypophysiotropic neurons are responsible for releasing CRF into the HPPVs. Hypophysial (pituitary) portal vessels vascularize the anterior pituitary and transport CRF to interact with a specific G protein-coupled receptor (CRF-R1), resulting in stimulation of the synthesis of pro-opiomelanocortin (POMC), an ACTH precursor peptide, as well as stimulation of secretion of ACTH and other POMC-derived peptides (Turnbull and Rivier, 1997; Vale *et al.,* 1981).

The adrenal glands are situated on top of each kidney, consisting of two layers of cells - an adrenal medulla and an adrenal cortex, each of which function as endocrine glands (Vander, 2001). The three distinct layers of the cortex are: (a) zona glomerulosa – the outer cells of the cortex which predominantly produce aldosterone from corticosterone since it lacks the enzymes necessary to form cortisol and androgens; (b) zona fasciculata - the middle cortex layer that produces no aldosterone but mainly cortisol and androgens; and (c) zona glomerulosa – the inner cortex layer that also produces no aldosterone but mainly cortisol and androgens (Vander, 2001). All of the adrenal steroid hormones are produced in the adrenal cortex from the steroid-precursor

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cholesterol, most of which is provided by the plasma (Vander, 2001). A simplified illustration of the steroid biosynthesis cascade is shown in Fig. 2.2. The five adrenal cortex hormones of high physiological significance are aldosterone (the most important mineralocorticoid), cortisol (the most important GC in humans), corticosterone (the most important GC in rats and mice), dehydro-epiandrosterone (DHEA, an androgen) and androstenedione (also an androgen). Since our interest is mainly in the GCs, cortisol and corticosterone, the other hormones will not be discussed. ACTH potently stimulates the synthesis and subsequent release of GCs from the zona fasciculata (Keller-Wood and Dallman, 1984; Young *et al.,* 1986).

The inner adrenal medulla secretes catecholamines (which form part of the first wave of hormonal responses to stress – see figure 2.1 and section 2.1.2) (Vander, 2001). Actually, the adrenal medulla is a modified sympathetic ganglion with cell bodies devoid of axons.

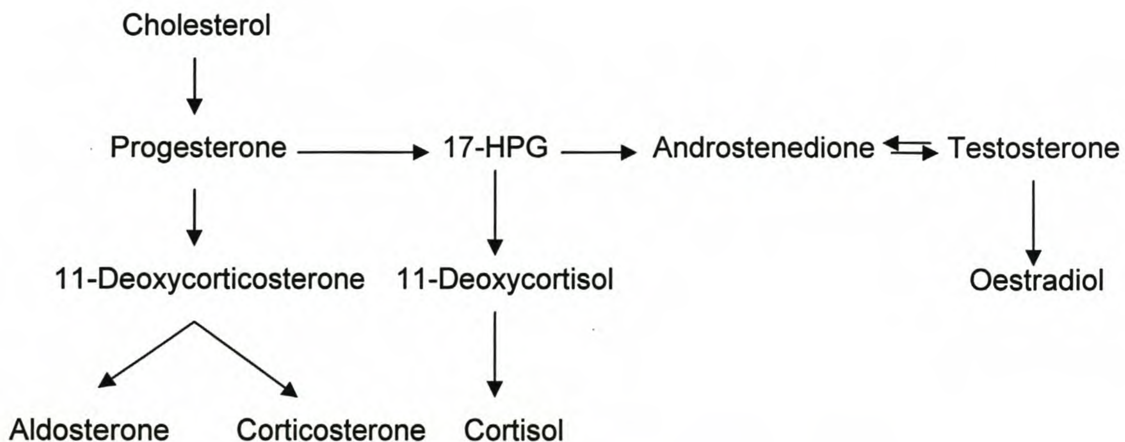

Figure 2.2 A simplified scheme showing adrenal steroid biosynthesis. Cholesterol is the common precursor for all the different steroids. Cortisol is the major GC in humans, with corticosterone being its equivalent in lower species such as rats and mice. Abbreviation: 17-HPG, 17-hydroxyprogesterone. Adapted from Harvey, 1996.

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Instead, the cell bodies release their secretions into the blood, thereby fulfilling a criterion for an endocrine gland (Vander, 2001). It is this direct neural activation for release that causes one of the major differences between catecholamine and GC response time.

2.2.2 Glucocorticoid transport in plasma

Steroid hormones (such as GCs) are lipid-soluble and, once synthesized, easily diffuse into the blood where they mainly bind to certain plasma proteins (Vander, 2001). For example, GCs in the blood bind mainly to corticosteroid binding globulin (CBG), which is the major transport protein for GCs in the blood of most vertebrate species (Seal and Dou, 1965). In fact, >90% of the cortisol (human) and corticosterone (rat) in plasma is bound by CBG, leaving a small fraction evenly distributed between albumin and a pool of non-protein bound or "free" steroid that is generally assumed to be biologically active (Siiteri *et al.,* 1982; Brien, 1981). CBG, therefore, plays a major role in cortisol metabolism by regulating the hormone's bioavailability and metabolic clearance (Bright, 1995; Ousova *et al.,* 2004).

CBG is a well-conserved α-glycoprotein in vertebrates. It is synthesized in the liver and secreted into the blood where it binds cortisol and progesterone with a high affinity (K_A ~ 10 nM^{-1}) (Ousova *et al.,* 2004). Analysis of its molecular structure reveals that CBG belongs to the serine protease inhibitors and substrates (SERPINS) superfamily and therefore does not share any sequence homology with other steroid-binding proteins (Carrell and Travis, 1985; Hammond *et al.,* 1987; Hammond *et al.,* 1990). Pemberton *et al.* showed that CBG is a substrate of the serine-protease elastase, which cleaves CBG near its steroid binding site resulting in the local release of cortisol at sites of inflammation (Pemberton *et al.,* 1988).

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Membrane receptors for CBG have also been described (Strel'chyonok and Avakumov, 1991). These membrane receptors capture the CBG-cortisol complex before transporting it into the cell where it subsequently dissociates (Strel'chyonok and Avakumov, 1991). In contrast to previous perceptions, this finding suggests that it is not only the free steroid that shows biological activity. *In vitro* studies showed that after the binding of the CBG-cortisol complex to its receptors, cAMP increased in the cell – suggesting that CBG itself may have intrinsic biological properties (see review of Breuner and Orchinik, 2002). More than 68% of CBG in rats remain in the cortisol-free state under physiological conditions, supporting the hypothesis that CBG may also act as a hormone (Gayrard *et al.,* 1996). This, however, deserves no further elaboration since it does not fall under the scope of interest of this thesis.

2.2.3 Molecular mechanisms of glucocorticoid action

Free or biologically active GCs diffuse into target cells where they exert a plethora of molecular actions. Most effects of GCs, if not all, are mediated via the Type 2 glucocorticoid receptor, the GR, a 777 amino acid protein cloned in humans in 1985 (Hollenberg et al., 1985). The GR is a member of the superfamily of ligand regulated nuclear receptors, which all contain modular structures whose principal functions (ligand binding, DNA binding and activation) are localised to specific domains (Fig. 2.3) (Giguere et al., 1986; Hollenberg and Evans, 1988; Dahlman-Wright et al., 1995; Beato M et al., 1996).

In humans, GRs exist in two isoforms, GRα and GRβ, as a result of alternative mRNA splicing (Bamberger et al., 1995). In mice, however, no GRβ has been detected and, therefore, the effects of GCs are mediated solely through binding to GRα (Otto *et al.* 1997). GRβ mRNA was found in rats, specifically in gastrocnemius muscles (Korn *et al.,* 1998).

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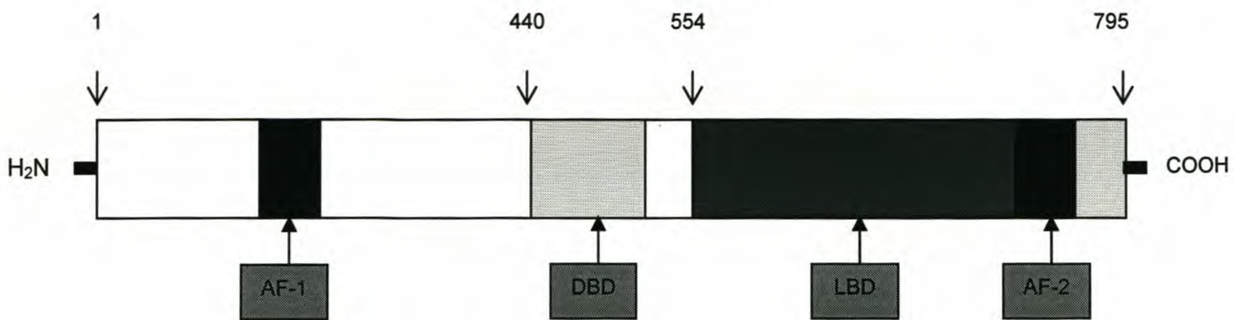

Figure 2.3 A linear representation of the structural organization of the rat glucocorticoid receptor (GR). Specific domains contain sites responsible for the principle functions, viz. activation, ligand-binding and DNA-binding. *Abbreviations:* AF-1 and AF-2, binding sites for activation factors 1 and 2; DBD, DNA-binding domain; LBD, ligand-binding domain; H_2N, amino terminal; COOH, carboxy terminal.

Although the physiological significance of GRβ-expression is still unknown (Pujols *et al.,* 2002), it is defective in steroid binding and therefore acts as a dominant negative inhibitor of GRα function (Bamberger et al., 1995; Oakley et al., 1999). However, controversy exists regarding the level of expression of GRβ, which may be due to methodological differences. Researchers who found that GRβ has low abundance in human cells and tissues suggest it is unlikely to have any effect on GRα function (Pujols *et al.,* 2002).

When no ligand is present, GR is predominantly maintained in the cytoplasm of the target cell, bound in an inactive multi-protein complex consisting of two heat-shock protein (hsp)-90 molecules and a number of other molecules which include, amongst others, immunophilins p59 and calreticulin (Fig 2.4) (Beato *et al.,* 1996). Ligand presence and subsequent binding to the ligand-binding domain (LBD) of the GR lead to a conformational change in the receptor, which causes dissociation of the multi-protein complex (Beato, 1989).

Nuclear translocation (movement from the cytoplasm into the nucleus) of the GR occurs by virtue of the nuclear localization sequence within the DNA binding

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domain (DBD), which is exposed upon ligand-induced conformational change (Beato, 1989). In the nucleus, GR binds to DNA sequences in the promoters of GC-responsive genes referred to as glucocorticoid response elements (GREs), to activate or repress transcription of these responsive genes. These activating or repressive mechanisms are known as transactivation and transrepression, respectively.

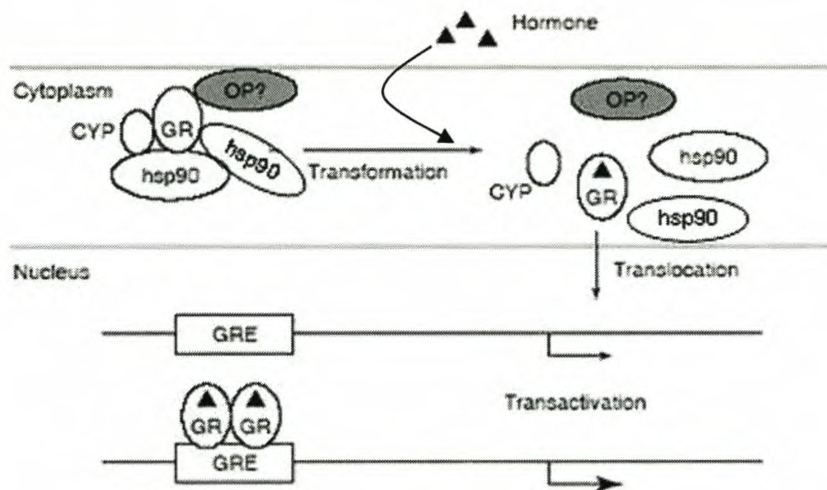

Figure 2.4 The glucocorticoid receptor signal transduction cascade. Glucocorticoid hormones enter the cell, bind to glucocorticoid receptor (GR). The bound ligand-receptor complex translocates to the nucleus and binds to glucocorticoid response elements in the nucleus, causing either transactivation or transrepression. *Abbreviations:* CYP, cyclophilins; GRE, glucocorticoid response element; HSP, heat shock protein; OP?, other proteins (some of which were identified, e.g. immunophilin p59 and calreticulin) (adapted from Webster and Cidlowski, 1999).

GR cooperatively binds classical GRE sites (Fig 2.5A) as a homodimer, i.e. two identical activated GR molecules that have dimerized, to transactivate genes (Newton, 2000). As an example, after ligand binding, a GR homodimer activates transcription of the tyrosine aminotransferase (TAT) gene (section 2.3.1) - by binding to a DNA sequence upstream of the TAT-gene promoter (Jantzen *et al.,* 1987). Also, it has been shown more recently that GCs inhibit transcriptional

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repression by binding to two GREs in the GS gene promoter (Chandrasekhar *et al.,* 1999) (see section 2.3.2 for more details). Other modes of transactivation that do not require dimerisation of the GR have been proposed such as the composite GRE (Fig 2.5B) and the tethering GRE (Fig 2.5C). An example of composite GR binding is found in the ability of GR to act synergistically either with activator protein-1 (AP-1), a transcription factor, or its components, c-Fos and c-Jun (Pearce and Yamamoto, 1993). Stat5 is a transcription factor that synergistically binds with GR, without the requirement of GR-DNA-binding, which is an example of tethered transactivation (Stocklin *et al.,* 1997).

In contrast, GRs can also cause transrepression of genes via direct binding to a negative GRE (nGRE) site (Figs. 2.5D-E, G) (Beato, 1989). The exact mechanisms for the different modes of repression have not been characterized yet, but speculations are that they may involve either protein-protein interactions with other factors on the promoter or direct inhibition due to steric hindrance as a result of the close proximity to the TATA box and transcription start site. Also, binding of GR to nGRE may block binding of positive factors and thereby cause transcriptional repression (Fig. 2.5G) (Newton, 2000). Examples of such cases, in which transrepression is caused via DNA-binding of homodimeric GRs to nGREs, are GR- inhibition of the POMC-gene (Figs 2.5D) and the glycoprotein hormone α–subunit gene (Fig 2.5G). Furthermore, Newton summarizes other mechanisms of transrepression caused by GR, which involve indirect DNA-binding (Newton, 2000). It has also been shown that monomers of GRs can repress transcription of genes using a tethering mechanism (Fig. 2.5F), such as the interaction between GR and the AP-1 and NF-κB transcription factors (Yang Yen *et al.,* 1990). The tethering mechanisms have been proposed to account for the GC-dependent repression of genes that do not have nGRE sites in their promoters, e.g. the inflammatory genes (Newton, 2000).

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Inflammatory protein genes, such as IL-8, IL-6 and IL-1, are the genes activated by the transcription factors NF-κB and AP-1 (nuclear factor-κB and activator protein-1). The ubiquitous NF-κB protein is a heterodimer, typically consisting of the p50 and p65 monomeric proteins (amongst others); the AP-1 protein is composed of either homo- or heterodimers between members of the Jun (e.g. c-Jun, Jun-B), Fos (e.g. c-Fos, Fos-B), activating transcription factor (e.g. ATF-2, B-ATF) or Maf (e.g. c-Maf, Maf-B) families (De Bosscher et al., 2003).

GRs negatively regulate transcription by direct or indirect interference with the transcriptional activity of the NF-κB and AP-1 transcription factors. Indirect interference occurs because NF-κB is sequestered in the cytoplasm with IκBα, an inhibitor, whose degradation is induced by inflammatory stimuli (Saklatvala, 2002; Scheinman *et al.,* 1995; Auphan *et al.,* 1995). GR-binding to the DNA causes increased synthesis of IκBα, which binds activated NF-κB, thus limiting its effects (O'Connor *et al.,* 2000; Auphan *et al.,* 1995).

Having discussed the mechanisms of GC action, including alluding to the mechanisms for upregulation of TAT and GS, it will be most appropriate to discuss these GC-inducible enzymes in more detail in the following section.

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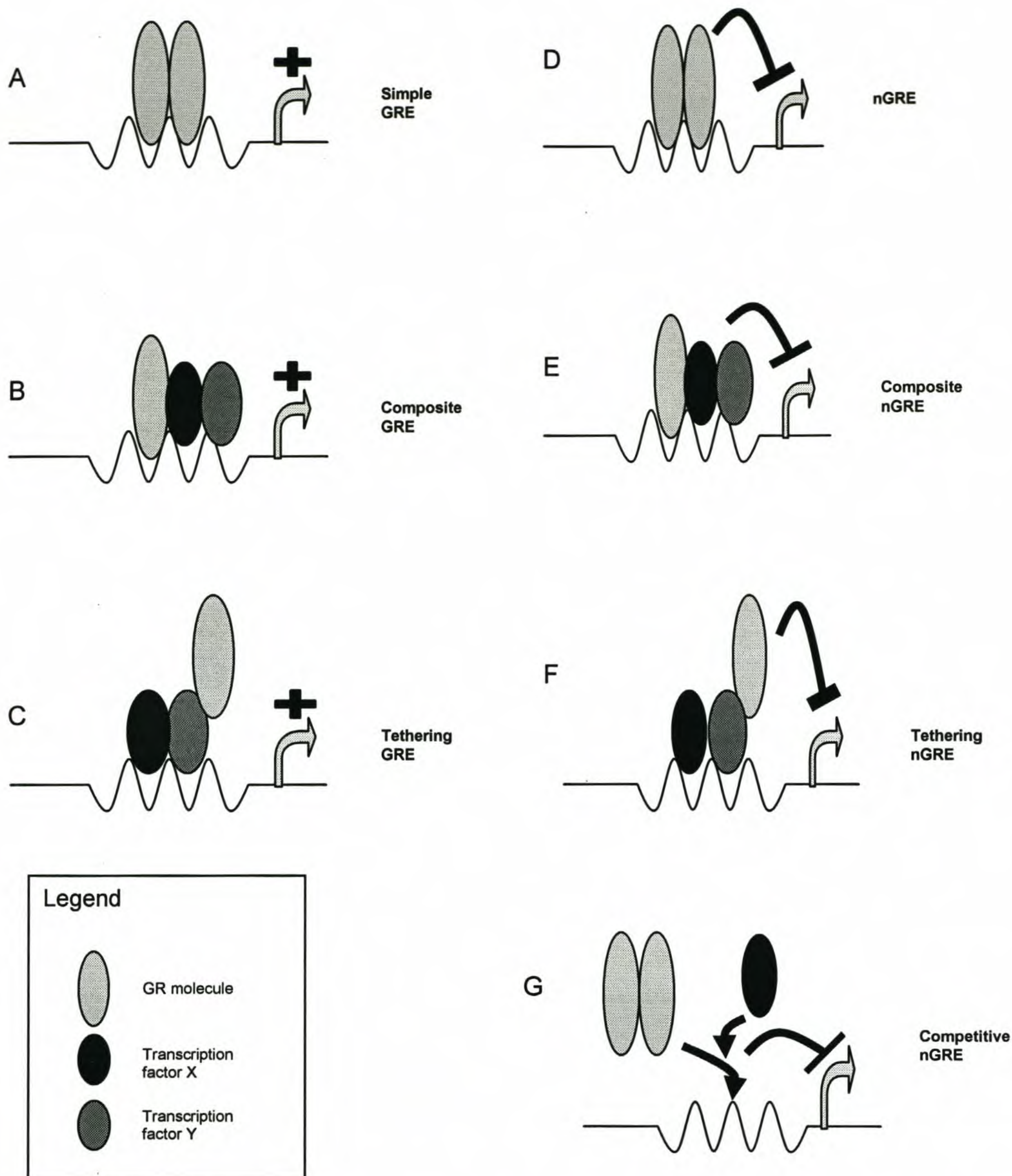

Figure 2.5 Models describing the molecular action of activated GR. (A) Homodimers of GR bind cooperatively to classical GRE sites to activate transcription. (B) Interaction of GR with a second transcription factor can activate transcription from composite binding sites in a manner that involves DNA binding of both factors. (C) Interaction of GR with a second transcription factor may result in activation of transcription in a manner that does not require DNA binding of GR. (D) Homodimers of GR repress transcription from a simple nGRE. (E) Interaction of GR with a second transcription factor can repress transcription from composite binding sites in a manner that involves DNA binding of both factors (F) Interaction of GR with a second transcription factor may result in repression of transcription in a manner that does not require DNA binding by GR. (G) At a competitive nGRE, binding of GR to the GRE site prevents binding of factors that are required for transcriptional activation and therefore causes transcriptional repression (Newton, 2000).

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2.2.4 Glucocorticoid-responsive metabolic enzymes

As part of the "adaptive redirection of energy" that occurs with physical adaptation during stress, the body increases gluconeogenesis and lipolysis (Chrousos & Gold, 1992). Historically, GCs derive their name from their involvement in increasing blood glucose levels. Current understanding of this scenario includes a view that GCs mediate the control of gene expression of genes involved in gluconeogenesis, amongst other processes. Of relevance to this thesis are the genes that encode for the metabolic enzymes, GS and TAT, which both contain GREs, which are binding sites for activated glucocorticoid receptors. As will be explained in the next two sections (2.2.4.1 and 2.2.4.2), these enzymes are involved in the metabolism of amino acids, which will ultimately serve as precursors for gluconeogenesis in the liver. Due to the fact that the activities of both these enzymes are under glucocorticoid control, their responsiveness during chronic stress represents our focus on the metabolic changes that occur during psychological stress.

2.2.4.1 Tyrosine aminotransferase (TAT)

Tyrosine aminotransferase (L-tyrosine: 2-oxo-glutarate aminotransferase, EC 2.6.1.5, TAT) converts tyrosine to p-hydroxyphenylpyruvate in a transamination reaction, and thereby catalyzes the first step in the catabolism of tyrosine (Cannellakis and Cohen, 1956). TAT is found in many organs throughout the body, including the liver, thyroid, heart, kidney, muscle, cerebellum, cerebrum, skin and adipose tissue (Prieur *et al.,* 2001). TAT activity was shown to increase several fold in rat liver after administration of GCs and since then the liver has become the main organ in which TAT was studied (Lin & Knox, 1958).

Corticosteroid concentrations, and subsequently TAT activity, increase after exposure to stress (Al-Mohaisen *et al.,* 2000). Regulation of the TAT gene, *Tat*, involves two remote glucocorticoid responsive units (GRUs), referred to as HSIII

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and HSV, which are located 2.5 and 5.4 kb upstream from the transcription start site, respectively (Grange *et al.,* 1989). Hormone-response units are clusters of transcription factor-binding sites, comprising a hormone-responsive element and a number of *cis*-elements (accessory factors). Such a hormone-response unit allows the regulation of transcription of the gene in space and time by integrating multiple signal pathways (Schöneveld *et al.,* 2004; Mitchell *et al.*, 1994). TAT GRUs consist of numerous contiguous and overlapping binding sites for the GR (viz. the GRE) and other transcription factors, including members of the C/EBP (CCAAT/enhancer-binding protein), HNF-3 (Hepatic Nuclear Factor 3) and Ets families of transcription factors (Jantzen *et al.,* 1987; Grange *et al.,* 1991; Espinás *et al.,* 1994; Roux *et al.,* 1995). Grange *et al.* showed that cell type-specific GC response in rat hepatoma cells is associated with binding sites for the cell type-specific nuclear proteins in TAT gene GRUs (Grange *et al.,* 1991). Full glucocorticoid induction of the rat TAT gene is achieved through cooperative interaction of the remote GRUs (Grange *et al.,* 1989). HSIII contains three GR binding sites, known as GRE 1, 2 and 3, of which GRE 2 and 3 are located within regions necessary for HSIII activity (Jantzen *et al.,* 1987). HSV contains a GR binding site essential, but not sufficient, for its activity since it lacks flanking sequences (Grange *et al.,* 1991). HSV is non-active in the absence of HSIII (Grange *et al.,* 1991).

Of particular relevance to this thesis is the TAT gene and the mechanism for its transactivation by GR. It has been shown that the GR uses a dynamic multistep mechanism to recruit accessory DNA binding proteins that assist in the activation process of the TAT gene (Grange *et al.,* 2001). The three successive steps are (i) chromatin remodeling, (ii) DNA demethylation and (iii) synthesis of accessory factors (Grange *et al.,* 2001). The accessory factors are the proteins that contribute slightly but additively to the activity of the GRU (Grange *et al.,* 2001). A typical GRU will consist of one or more GREs with additional binding sites for related transcription factors such as C/EBP (Grange *et al.,* 2001). A stable

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multiprotein complex is formed, which interacts with the regulatory element - then only can efficient transcriptional induction be achieved (Grange *et al.,* 2001).

Apart from GCs, insulin and glucagon also control TAT activity (Holten & Kenney, 1967; Ernest & Feigelson, 1979). Earlier research has shown that insulin induces TAT mRNA and directly influences TAT activity (Reel *et al.,* 1970; Spencer *et al.,* 1978), whereas, in the presence of glucocorticoids, insulin acts at a post-transcriptional level only increasing TAT activity (Crettaz *et al.,* 1988). However, more recent research has shown that insulin has a negative effect on genes that contain a PEPCK-like motif (a type of motif that defines an insulin response sequence, IRS), which includes the gene that expresses TAT (O'Brien *et al.,* 2001; O'Brien & Granner, 1996). It has also been shown that insulin inhibits gluconeogenesis (Ganss *et al.,* 1994). In contrast, glucagon binds to cell surface receptors and activates a second messenger system (cAMP), which leads to phoshorylation and activation of a variety of transcription factors that up-regulate TAT expression and hence activity (Schmid *et al.,* 1987).

Glucocorticoid-induction of TAT has been investigated in various *in vitro* models, e.g. TAT induction by dexamethasone phosphate in cell culture (Granner *et al.,* 1968). It was shown that TAT accumulation on induction is due to an increase in its rate of synthesis (Granner *et al.,* 1968). Also, studies on the hepatic tissue culture (HTC) system have suggested that dexamethasone stimulates both the accumulation of TAT mRNA and its translation into protein (Peterkofsky & Tomkins, 1967; Tomkins *et al.,* 1966). During 1970, a report by Granner *et al.* concluded that steroid presence is constantly required for maintenance of the induced rate of synthesis (Granner *et al.,* 1970). More recently, the *in vivo* effects of chronic methylprednisolone (MPL)-infusion in adrenolectomised rats were investigated (Ramakrishnan *et al.,* 2002; DuBois *et al.,* 1995). An early rise in TAT activity was found after 6 hours of MPL treatment, but this was followed by a dramatic decrease which stabilized at values close to control values after 24

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hours of MPL treatment (Ramakrishnan *et al.,* 2002). However, the exact mechanisms of these effects remain unclear.

The effects of cytokines on TAT activity have not received much attention over the past fifteen years. In an earlier report, the interaction between cytokines and TAT activity were investigated in rat hepatoma FAO cells and it was found that the cells in the IL-6-containing media increased their TAT activities (Guzdek *et al.,* 1995). More recently, it was shown an IL-6 chimera (IL-6/soluble IL-6 receptor) was able to dose-dependently reduce the expression of tyrosine aminotransferase in fetal hepatocytes (Zvibel *et al.,* 2004). Based on these conflicting reports, it became of interest to us to determine what effects cytokine action would have on TAT expression *in vivo.*

Furthermore, a diurnal variation in TAT enzyme activity has been reported, which, according to the authors, appeared to be independent of adrenal activity, an assessment which appears to be contradictory to their results, as illustrated in Fig 2.6 (Wurtman and Axelrod, 1967). However, it has since been shown that GCs, as explained before, regulate TAT enzyme expression. Yi-Li *et al.* showed that there is a good correlation between the level of GR and induction of TAT in rat liver, and that down-regulation of GR led to a decrease in TAT induction (Yi-Li *et al.,* 1989). At the level of activity-expression relationship, DuBois *et al.* showed that the profile of TAT activity exhibits similarities to the profile of TAT mRNA expression (DuBois *et al.,* 1995), i.e. a rise in TAT activity is concomitant with an increase in TAT mRNA levels.

2.2.4.2 Glutamine synthetase (GS)

Glutamine synthetase (GS; L-glutamate:ammonia ligase (ADP-forming); EC 6.3.1.2.) is a “housekeeping” enzyme that is expressed at a particularly high level in neural tissues (Grossman *et al.,* 1994; Patejunas and Young, 1987), skeletal muscle and lungs (Souba, 1987; Souba & Austgen, 1990). Muscle and lungs

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synthesize and release glutamine (Gln) into the blood (Souba, 1987; Souba & Austgen, 1990).

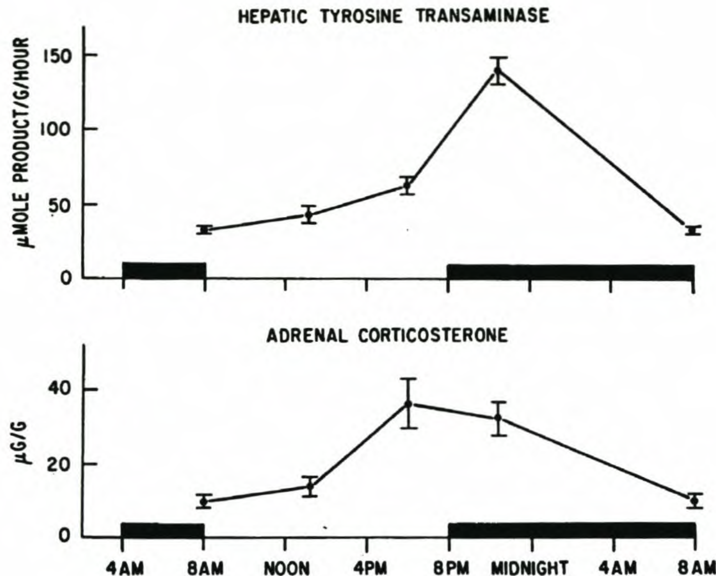

Figure 2.6 Daily rhythms in hepatic tyrosine aminotrasferase (TAT) and adrenal corticosterone content. The graph clearly demonstrates that increased TAT activity follows the rise in adrenal corticosterone levels. Rats were kept under controlled illumination (lights on: 8 a.m. to 8 p.m.) for 1 wk prior to assay. Vertical lines in both figures represent standard errors of the mean (Taken from Wurtman and Axelrod, 1987).

Gln is the most abundant free amino acid in the blood and tissues of humans and rats (Bergström *et al.,* 1974; Ardawi & Jamal, 1990). The many physiological roles of this amino acid include substrate for renal ammoniagenesis (Welbourne *et al.,* 1986), precursor for nucleotide biosynthesis (Austgen *et al.,* 1992), regulator of muscle protein turn-over (Rennie *et al.,* 1989) and vehicle for nitrogen-transfer between tissues (Souba, 1987). Glutaminase is the other key enzyme involved in Gln metabolism – it catalyses the hydrolysis of Gln to Glu (Souba *et al.,* 1985). Enterocytes, lymphocytes and endothelial cells consume

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Gln in large amounts and have more glutaminase than GS (Souba *et al.,* 1990; Newsholme & Parry-Billings, 1990; Souba & Austgen, 1990).

In skeletal muscle, the conversion of muscle protein and amino acids into Gln provides an important source of Gln during stress, when there is often an increased demand or limited supply of it (Labow *et al.,* 1999). Glu is converted to Gln (GS-catalyzed reaction), which the muscle releases into the blood from where it is taken up by the kidney and cells of the immune system (Salway, 1994). Increased transcription in response to hormone action represents one of two major ways by which GS expression in mammalian systems is regulated (Labow *et al.,* 2001). The other manner of regulation of GS expression occurs through regulation of protein stability in response to Gln concentration (Labow *et al.,* 2001). As part of the acute stress response, GCs are rapidly released, which in turn up-regulate muscle GS gene expression *in vitro* and *in vivo* (Max *et al.,* 1988; Max, 1990). Also, muscle GS mRNA levels have been shown to rise in response to endotoxin challenge in a manner that is predominantly adrenal gland dependent (Lukaszewicz *et al.,* 1997). Muscle GS mRNA levels can be rapidly increased due to GCs during acute stress (Labow *et al.,* 1999). Chronic stress can cause Gln to impact directly on GS gene expression or potentiate the effects of another mediator; however, significant increases in GS gene expression can occur without changes in tissue Gln levels or circulating GCs (Labow *et al.,* 1999). GS expression in skeletal muscle tissue is regulated by both transcriptional and posttranscriptional mechanisms – Gln appears to affect GS expression (in muscle) differently during acute stress compared with chronic Gln and/or caloric deprivation (Labow *et al.,* 1999).

The rat GS gene contains two regions which were identified as conferring significant GC inducibility (Chandrasekhar *et al.,* 1999). One region is located far upstream (nearly 6 kb) from the transcriptional start site of the GS gene and the other is located in the first intron. A GRE is located in the intron region and three separate GRE half-sites are contained in the far upstream region (Labow *et al.,*

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2001). Current speculations are that GC regulation of GS expression is regulated by transcriptional repression in the absence of hormone caused by the two separate elements within the GS gene (Chandrasekhar *et al.,* 1999). In short, the GC inducibility of GS in lung and muscle may be due to relief from transcriptional repression, which does not operate in tissues (such as liver and kidney) with a higher basal expression of GS (Chandrasekhar *et al.,* 1999). Thus, GC-induced GS expression may explain the ability of lung and muscle to increase GS expression in catabolic states, e.g. exercise and stress (Chandrasekhar *et al.,* 1999). In addition, a more recent study has confirmed the existence of an inhibitory GRU in the GS gene, which showed GC-dependence in muscle cells and is located approximately 1.6 kb upstream from the transcriptional start site of the GS gene promoter (Garcia de Veas Lovillo *et al.,* 2003). This suggests that both positive and negative elements, as well as interactions between them, regulate GS promoter activity *in vitro* (Garcia de Veas Lovillo *et al.,* 2003).

Although the only known mediator of GS gene expression appears to be GCs, other factors (β-adrenergic agonists, cyclic AMP, epidermal growth factor, insulin and thyroid hormones) affect GS levels in the brain (de Vellis *et al.,* 1986; Khelil *et al.,* 1990; O'Bannion *et al.,* 1994; Jackson *et al.,* 1995). Chao *et al.* showed that IL-1β and TNF-α have no direct effect on GS protein levels while Huang and O'Bannion showed that IL-1β and TNF-α down-regulate the dexamethasone (dex)-induction of GS (Chao *et al.,* 1992; Huang and O'Bannion, 1998). No studies thus far have focused on peripheral GS expression in response to immobilisation stress in combination with an intervention that can probe the role of IL-6.

In keeping with the notion that GCs must "prevent the stress-response from overshooting" (Munck *et al.,* 1984), the concept of GC resistance must be discussed.

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2.3 Glucocorticoid resistance

'Glucocorticoid resistance' is the term that describes decreased target sensitivity to GCs (a decrease in GR expression), also associated with compensatory hypersecretion of glucocorticoids (Chrousos et al., 1993). Thus, elevated plasma concentrations of GCs are often used as an index of stress. Chronic stress (including psychological stress) and the attendant chronic hypersecretion of GCs have been linked to many different pathologies (Chrousos and Gold, 1992; Chrousos, 1995). The elaborate mechanisms responsible for maintaining GC homeostasis compensate for the insensitivity of tissues to glucocorticoids by resetting the hypothalamic-pituitary-adrenal axis. Thus, CRH, ACTH and cortisol secretion are increased. The compensatory increase in ACTH production causes increased secretion of GC precursors with mineralocorticoid activity (deoxycorticosterone, DOC; corticosterone, B) and increased secretion of several adrenal androgens (Fig 2.7) (Chrousos *et al.,* 1993). Although the GC concentrations in circulation is increased, they fail to exert their effects on the target tissues. Different molecular defects alter the functional characteristics or concentrations of the intracellular GR, which may be responsible for the development of glucocorticoid resistance (Chrousos *et al.,* 1993).

Several authors have shown that a single acute stress resulted in dramatic 3- to 4-fold elevation in plasma corticosterone levels (Omrani *et al.,* 1980; Sapolsky *et al.,* 1984; Meaney *et al.,* 1991; Alexandrova, 1994; Al-Mohaisen *et al.,* 2000). The last of these groups found that plasma corticosterone levels remained 2- to 3-fold above basal levels during persistent repeated immobilization stress of 30 days duration (Al-Mohaisen *et al.,* 2000).

GR availability (the amount of cytosolic GR that is available for binding to corticosteroid ligand) influences subsequent steroid responsiveness, which warrants the study of changes in GR levels during repeated stress. Due to inconsistent methodology, controversy exists in the literature regarding GR levels

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after exposure to chronic stress. Steroid unoccupied receptors have been measured by using radioligand binding assays and the decrease in cytosolic GR is often attributed to its translocation into the nucleus due to increasing corticosterone levels (Al-Mohaisen *et al.,* 2000).

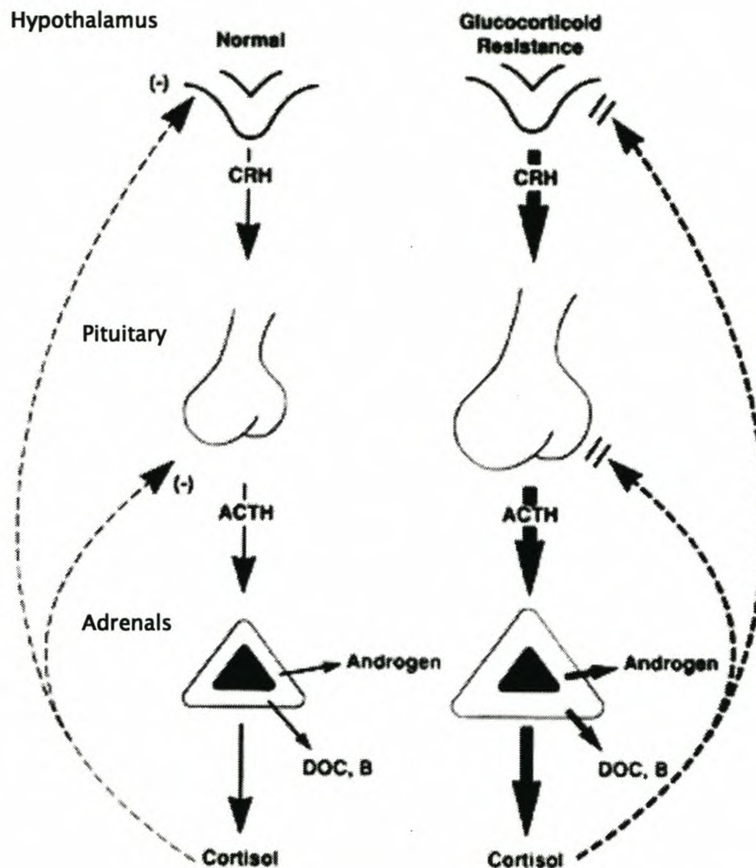

Figure 2.7 Pathophysiological mechanism of glucocorticoid (GC) resistance. With the normal physiological condition, the hypothalamus is sensitive to stimuli that promote the secretion of CRH. In contrast, subsequent GC resistance leads to hypersecretion of CRH, ACTH and cortisol, which desensitizes the hypothalamus to further endocrine stimuli. ACTH, adrenocorticotrophic hormone; CRH, corticotrophin releasing hormone; DOC, deoxycorticosterone; B, corticosterone (Adapted from Chrousos *et al.,* 1993).

However, in their article, Al-Mohaisen *et al.* also claim that their methodology which combined an unoccupied receptor assay and a total receptor exchange binding assay, measured true total GR for the first time. Their data suggested

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that previous studies, mostly using free receptor binding assays, underestimated the GR levels by not measuring steroid occupied GR during chronic stress (Al-Mohaisen *et al.,* 2000). They found an increase in total GR after exposure to chronic stress, in disagreement with what most studies on the issue have shown (reviewed in Al-Mohaisen *et al.,* 2000). Following their report, no study on chronic stress has supported this viewpoint, and no other researcher has tested this 'new' approach. We thus remain skeptical with regards to the feasibility of this new methodological approach.

Al-Mohaisen *et al.* found that unoccupied cytosolic GR decreased during both acute and repeated stress in the rat liver (Al-Mohaisen *et al.,* 2000). As mentioned before, they found that occupied and total cytosolic GR levels increased in liver during 7-30 days of repeated immobilization (Al-Mohaisen *et al.,* 2000). Prior to aforementioned study, a general interpretation held that cytosolic GR levels decreased after chronic stress (Sapolsky *et al.,* 1984; Reul *et al.,* 1990). Sapolsky *et al.*, in male Fisher 344 and Long–Evans rats, observed reduced GR number (assessed by radioligand receptor binding assay) in amygdala and hippocampus during repeated immobilization stress (Sapolsky *et al.,* 1984). Alexandrova and Farka reported that 15 days of repeated stress resulted in down-regulation of GR (assessed by radioligand receptor binding assay) in rat liver (Alexandrova & Farka, 1992). Swimming stress caused a dramatic increase in plasma corticosterone levels with a sharp decline in hepatic cytosolic GR levels (Omrani *et al.,* 1980). Two other groups found down-regulated GR expression in rat hippocampus during chronic stress, using *in situ* hybridisation and immunoautoradiography, respectively (Gomez *et al.*, 1996; Herman & Spencer, 1998). In contrast, Lowy found no changes in GR density of various tissues of rats after repeated immobilization stress for 5 days (Lowy, 1991). The conflicting reports may possibly be due to the different strains of rats used in these mentioned studies as well as methodological differences.

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Immunological approaches (e.g. immunoblotting, immunoreactivity, enzyme-linked immunoabsorbent assays) have also been used to measure GR protein levels. For instance, and in contrast to above-mentioned studies, significantly increased GR immunoreactivity in hippocampal subregions of chronically stressed rats was measured (Kitayama *et al.*, 1989; Biagini *et al.*, 1993). In addition, Curtis and Rarey, by using an enzyme-linked immunoabsorbent assay, reported elevation of GR levels in spiral ligament tissues of rats after 2 days of immobilization stress (Curtis and Rarey, 1995). Table 2.1 summarizes some of the different GR responses as discussed above.

Al-Mohaisen *et al.* proposed that their observed up-regulation of GR may be an adaptive and protective response for survival during prolonged and persistent stress (Al-Mohaisen *et al.* 2000). The majority of these studies have investigated GR expression in the brain, which does not help to understand the metabolic effects that stressors might have on the organism. In this respect we thought it would be most relevant to investigate peripheral GC action (plasma corticosterone levels; GR binding activity in liver and muscle) in response to psychological stress.

Following this discussion on GCs, the next section will shift its focus to cytokine physiology, which will ultimately lead to discussion of the cross-talk that exists between the cytokine and endocrine systems.

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Table 2.1 Summary of *in vivo* GR responses of rats to different stress paradigms

Authors, Date	Stressor(s) and Dose	Duration	Tissue	Type of GR assay	GR Result
Omrani *et al.*, 1980	swimming	2 min	liver	total GR exchange binding assay	down-regulation
Golikov *et al.*, 1981	IMO	48 h	liver	radioligand receptor binding assay	down-regulation
Sapolsky *et al.*, 1984	IMO	4 days	brain	radioligand receptor binding assay	down-regulation
Kitayama *et al.*, 1989	IMO	14 days	brain	immunocytochemistry	up-regulation
Gagnon *et al.*, 1987	IMO	not available	cardiac muscle	radioligand receptor binding assay	down-regulation
Lowy, 1991	corticosterone 10 mg/kg; IMO 2h/day	5 days	brain	radioligand receptor binding assay	Cort: down-regulation; IMO: no change
Alexandrova & Farkas, 1992	swimming or heat or cold or IMO or 10 mg/kg histamine; 500 mU/kg vasopressin	Acute; or 3x per day for 18 days	liver	radioligand receptor binding assay	down-regulation (after swimming and after histamine inj)
Biagini *et al.*, 1993	open field; noise and light	3 weeks	brain	immunocytochemistry	up-regulation
Cutis & Rarey, 1995	IMO 6 h/day	between 2 & 21 days	brain	ELISA	up-regulation
Gomez *et al.*, 1996	IMO	not available	brain	*in situ* hybridisation	down-regulation
Al-Mohaisen *et al.*, 2000	IMO 2 h/day	7, 15 or 30 days	liver	radioligand binding receptor assay; exchange assay; western blotting	down-regulation of free cytosolic GR; up-regulation of bound cytosolic GR & total cytosolic GR
Hu *et al.*, 2000	IMO 2 h/day OR DHEA: 5 mg/0.1 ml DMSO daily i.p.	60 days	liver, thymus, spleen	western blotting	IMO: up-regulation DHEA: down-regulation
Gursoy *et al.*, 2001	CR 30% of *ad libitum* diet with or without IMO 2 h/d	8 weeks	liver, thymus, heart, testis	western blotting	CR: down-regulation; CR + IMO: down-regulation
Sun *et al.*, 2002	sepsis by cecal ligation and puncture	acute	skeletal muscle	radioligand receptor binding assay	up-regulation

IMO, immobilisation stress; CR, caloric restriction; GR, glucocorticoid receptor; i.p., intra-peritoneal; s.c., sub-cutaneous; cort, corticosterone; ELISA, enzyme-linked immunosorbent assay

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2.4 Cytokine physiology

2.4.1 Definition

Cytokines are generally defined as 'regulatory proteins, secreted by white blood cells and a variety of other cells in the body, whose pleiotropic actions include numerous effects on cells of the immune system and modulation of inflammatory responses' (Thomson, 1991). Amidst many different ideas about what exactly a cytokine is, despite the definition of Thomson as shown above, the term 'cytokine' encompasses the 'monokines' (monocyte/macrophage-derived mediators) and 'lymphokines' (lymphocyte-derived mediators) (Turnbull and Rivier, 1999). Cytokines are large, soluble polypeptide mediators (with molecular mass ranging between 8 and 60 kDa) that regulate growth, differentiation and function of many different cell types (for a review see Turnbull and Rivier, 1999). Table 2.2 shows the main classes of cytokines with examples of each.

The arbitrary classification of cytokines into the different families remains controversial, but for purpose of this thesis focus is placed only on the IL-1 and IL-6 cytokine families. The remainder of this section will briefly discuss the properties of these cytokines and their actions, which will be followed by a proper account regarding the cross-talk between the endocrine and cytokine systems. I shall discuss mainly IL-1 and IL-6 since (1) they are closely linked and (2) the experimental part of this study is mainly interested in IL-6 and its action during the stress response.

2.4.2 Basic physiology of interleukins (IL-1 and IL-6)

The name 'interleukin' and the designation of IL-1 were coined at the 2nd International Lymphokine Workshop in Switzerland, 1979 (Di Giovine & Duff,

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1990). IL-1 has since been perceived as a major biological mediator in every organ system and is regarded as the 'prototypic' inflammatory cytokine (Di Giovine & Duff, 1990; Rothwell & Luheshi, 2000).

Table 2.2 Table showing different families of cytokines (from Turnbull and Rivier, 1999).

Family of Cytokines	*Members*
Interleukins	IL-1 to IL-18
Tumor necrosis factors	TNF-α, TNF-β
Interferons	IF-α, -β, -γ
Chemokines	IL-8/cinc/gro/NAP-1, MIP-1α, -β, RANTES
Hematopoietins (neuropoietins)	IL-6, CNTF, LIF, OM, IL-11, CT-1
Colony stimulating factors	G-CSF, M-CSF, GM-CSF, SCF, IL-3, IL-5
Neurotrophins	NGF, BDNF, GDNF, NT-3, NT-6
Growth factors	IGF-1, IGF-II, EGF, aFGF, bFGF, PDGF, TGF-α, TGF-β, activin

Abbreviations: aFGF, acidic fibroblast growth factor; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; cinc, cytokine-induced neutrophil chemoattractant; CNTF, ciliary neurotrophic factor; CT-1, cardiotropin-1; EGF, epidermal growth factor; G-CSF, granulocyte colony stimulating factor; GDNF, glial-derived neurotrophic factor; GM-CSF, granulocyte-macrophage colony stimulating factor; gro, growth-related oncogene; IGF, insulin-like growth factor; IL, interleukin; IL-1ra, IL-1 receptor antagonist; IFN, interferon; LIF, leukemia inhibitory factor; M-CSF, macrophage colony stimulating factor; MIP, macrophage inflammatory protein; NAP, neutrophil activating protein; NGF, nerve growth factor; NT, neurotrophin; OM, oncostatin M; PDGF, platelet-derived growth factor; RANTES, regulated upon activation normal and secreted; SCF, stem cell factor; TGF, transforming growth factor; TNF, tumor necrosis factor.

IL-1 is a family of three closely related proteins that are products of separate genes (Rothwell & Luheshi, 2000). IL-1α and IL-1β are the agonists and are believed to exert identical actions via binding to a single 80 kDa cell surface receptor, interleukin-1 receptor (IL-1R) (Sims *et al.,* 1988). When activated by binding of IL-1, the receptors initiate signaling pathways that lead to the release of secondary substances (e.g. prostaglandins and nitric oxide) that mediate inflammation and tissue remodeling (Dinarello, 2000). IL-1 receptor antagonist

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(IL-1RA) is the third member of the IL-1 family and is a naturally occurring cytokine in the IL-1-family of cytokines whose primary function is the prevention of any biological response that may occur as a result of IL-1 binding (Dinarello, 2000). Thus, the ratio of IL-1 to IL-1RA determines the net biological response elicited by the cytokin-receptor-complex.

IL-6 is a pleiotropic pro-inflammatory cytokine, produced by cells of the immune system (Van Snick, 1990; Akira *et al.,* 1990) as well as by cells in neuronal and endocrine tissues, such as the hypothalamus (Schobitz *et al.,* 1993; Muramani *et al.,* 1993), the anterior pituitary (Spangelo *et al.,* 1990) and the adrenal cortex (Judd & McLeod, 1992). IL-6 plays important roles in immune function (Van Snick, 1990; Akira *et al.,* 1990; Hirano *et al.,* 1990), hepatic acute phase protein synthesis (Heinrich *et al.,* 1990; Geiger *et al.,* 1998), HPA axis activation (Navarra *et al.,* 1991; Lyson & McCann, 1991; Perlstein *et al.,* 1993) and emotional behaviour (Butterweck *et al.,* 2003). Based on this range of functions, IL-6 is thought to be a hormone (Manfredi *et al.,* 1998). In addition, IL-6 exerts several actions on the CNS and is implicated in development of neural and development disorders (Hüll *et al.,* 1996).

IL-6 belongs to a family of cytokines that all interact with the common transmembrane cell-surface protein, gp130 (Turnbull & Rivier, 1999). In contrast to most other classes of receptors, the cytoplasmic domain is not necessary for proper signal transduction (Arzt, 2001). IL-6 binds to IL-6Rα (the α subunit of the functional receptor), which triggers the association of IL-6Rα with gp130 (Arzt, 2001). The association of the IL-6/IL-6Rα complex with gp130 leads to the homodimerization of gp130, which is a prerequisite for signal transduction through the IL-6R complex (Arzt, 2001). IL-6Rα is an IL-6-specific receptor and is therefore responsible only for binding of its ligand, IL-6 (Turnbull & Rivier, 1999).

In the adrenal glands, IL-6 receptor-α (IL-6Rα) mRNA has been detected mainly in zonas glomerulosa and fasciculata, as well as in the adrenal medulla

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(Cunningham *et al.,* 1992; Gadient *et al.,* 1995; Path and Bornstein, 1997). These data confirm the possibility of communication between IL-6 and GCs, but do not shed light on the exact mechanism or result of such communication. In the following section I will discuss evidence for cross-talk between cytokines and GCs. However, since the adrenals do not stand alone in the stress response, the discussion will, once again, begin with the elements of the HPA axis. Also, since it is difficult to discuss cross-talk between cytokines and GCs focusing only on IL-6, the discussion will include literature regarding IL-1, since more is known about IL-1 with relation to psychological stress.

2.5 Bi-directional interaction between endocrine and cytokine systems

Immune system activation in response to e.g. infection or other stressors leads to a classic stress response by activation of the HPA axis and sympathetic nervous system (SNS) (Maier, 2003). Numerous types of injury, infection, disease and/or psychological conditions are associated with increased production of endogenous cytokines that appear to increase concomitantly with elevations in HPA axis activity: head trauma, cerebral ischemia (stroke), autoimmune diseases, psychiatric and dementia disorders, AIDS and antigenic challenges (Turnbull and Rivier, 1999). The brain controls both HPA and SNS activity, therefore, the 'sickness pattern' can also be seen to include a centrally coordinated response (Maier, 2003; Kluger *et al.,* 1996).

Glucocorticoids and catecholamines, as mentioned earlier, are the endpoints of these systems - they function by e.g. promoting production and release of energy by means of stimulating the conversion of glycogen to glucose as well as the breakdown of protein into amino acids, which are used in gluconeogenesis. The brain is important in regulating the roles of peripheral organs (the main peripheral organs involved in energy metabolism are the liver and skeletal muscles) and

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immune cells during host defense, hence the existence of communication between the brain and the immune system (Maier, 2003). Immune system organs, such as the thymus, spleen, lymph nodes, gut-associated lymphoid tissue (GALT) and bone marrow, are innervated by terminals of the SNS (Felten *et al.,* 1985) and cells of the immune system express receptors for catecholamines (Sanders *et al.,* 2001) as well as for hormones of the HPA axis and other hormones (Miller *et al.,* 1998).

Despite the number of examples, direct and clear evidence for the involvement of particular cytokines in the HPA axis response to such insults has been limited to only a few cases, viz. viral or bacterial infection, local tissue damage and inflammation and acute physical/psychological stress. Also, the cytokine response to a stimulus that does not result in tissue damage or infection is quick (within minutes), suggesting a role for cytokines in homeostasis that has only recently received attention (Turnbull and Rivier, 1999). Direct demonstration of cytokine involvement in physiological or pathophysiological HPA axis responses has been restricted largely to IL-1, IL-6 and TNF-α.

2.5.1 Cytokine production in response to stress

Cytokines seem to be at the interface between the cognitive perception of the stressor and its physiological sequelae (Wilson *et al.,* 2002). Proof of this is provided by studies showing that administration of cytokines produces alterations in monoamine neurotransmitter systems that resemble those seen in states of stress (Connor and Leonard, 1998; Merali *et al.,* 1997; Song *et al.,* 1999). For example, systemic administration of IL-1 caused a modest increase in release of NE (in both the nucleus accumbens and the hippocampus) and dopamine (DA, in the hippocampus) (Merali *et al.,* 1997). In the same study, a mild neurogenic stressor was applied, which led to even greater increases in NE and DA release,

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as well as an increase in serotonin release (Merali *et al.,* 1997). Rats subjected to immobilisation stress increased their expression of hypothalamic IL-1β, which was followed by a progressive rise in the serum IL-6 level, thus indicating that hypothalamic IL-1 plays a pivotal mediating role in the stress-induced peripheral IL-6 production (Ishikawa *et al.,* 2001).

Studies during the last 15 years have shown that synthesis and/or secretion of IL-1 and IL-6 are altered during acute physical or psychological stress (e.g. LeMay *et al.,* 1990; Mekaouche *et al.,* 1994; Minami *et al.,* 1991; Shintani *et al.,* 1995a; Shintani *et al.,* 1995b; Zhou *et al.,* 1993). The sites of synthesis and/or secretion assessed in these studies ranged from the midbrain to the hypothalamus, as well as peripheral sites (the latter will be discussed in more detail in section 2.5.3). As a result, physical or psychological stress increases circulating IL-6 concentrations (LeMay *et al.,* 1990; Tataki *et al.* 1994; Zhou *et al.,* 1993). Experimental models that have resulted in elevated plasma IL-6 levels in rats include exposure to a novel environment, conditioned aversive stimuli, electroshock or restraint (LeMay *et al.,* 1990; Morrow *et al.,* 1993; Turnbull and Rivier, 1995a; Zhou *et al.,* 1993). The time course of this response is variable, depending in part on the type of stress. IL-6 mRNA is elevated in the midbrain and IL-6R mRNA is diminished in the midbrain and hypothalamus 4-24 h after restraint stress in the rat (Shizuya *et al.,* 1997). In mice, exposure to immobilization stress for 30 minutes caused elevated hepatic and splenic IL-6 mRNA expression, followed by an increase in serum IL-6 concentrations (Kitamura *et al.,* 1997). The increases in serum IL-6 levels after physical and/or psychological stresses in rats are much more rapid (within 15 min) than those observed after either local or systemic inflammations (1-3 h) and appear to be mediated by the actions of catecholamines (De Rijk *et al.,* 1994; Soszynski *et al.,* 1996; Takaki *et al.,* 1994; Van Gool *et al.,* 1990). Increases in blood levels of IL-6 do not appear to directly contribute to the HPA axis response to acute stress because, even though IL-6 levels increase rapidly, they still lag behind plasma ACTH responses (Turnbull and Rivier, 1995a; Zhou *et al.,* 1993). Table 2.3

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summarizes the effects that different stressors have on the *in vivo* production of IL-6.

However, the possibility of some communication between IL-6 (or other cytokines) and the adrenals is still a topic of research. In animal models, stressors such as acute restraint stress, immobilisation or tail shock induces hypothalamic IL-1β and IL-1β mRNA expression within 30 min of commencement of stress (Minami *et al.,* 1991; Suzuki *et al.,* 1997; Shintani *et al.,* 1995b). Chronic exposure to physical or psychological stress elevates plasma IL-1β concentrations in rats and humans and hypothalamic IL-1β mRNA in mice (Mekaouche *et al.,* 1994; Spivak *et al.,* 1997; Tagoh *et al.,* 1995). These models of chronic exposure lasted from 4 days to a few years after exposure to traumatic and other stressful events.

2.5.2 Cytokine production after dexamethasone and CRH treatment

Dexamethasone does not influence either basal or IL-1β-induced IL-6 secretion in zona glomerulosa cells (Judd and MacLeod, 1992). These authors also showed that ACTH administration increases release of IL-6 from zona glomerulosa cells, but not from zonas fasciculata or reticularis cells, despite similar ACTH-stimulated cAMP levels in each cell type – suggesting, at that stage, that HPA axis activation may cause IL-6 secretion from the adrenal (Judd and MacLeod, 1992). More recently, it was shown that IL-6 release from the adrenals during inflammation is CRH-dependent (Venihaki *et al.,* 2001). Also, peripheral CRH induces NF-κB DNA-binding in mouse thymocytes, providing proof that CRH fulfills a pro-inflammatory function (Zhao & Karalis, 2002), supporting the idea that not only do inflammatory agents influence HPA axis function (as discussed in the ensuing section), but that the HPA axis may in turn also regulate inflammatory actions during the stress response (thus fulfilling a proinflammatory function). It has been shown that GCs inhibit the production of

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Table 2.3 Summary of the effects of different stressors on the *in vivo* production of IL-6 in rodents

Author, Date	Experimental Model	Stressor(s)	Duration and Dose	Time of sacrifice after stress	Parameter measured, tissue	Assay details	IL-6 results
LeMay *et al.*, 1990	rats	psychological stress (open field)			plasma IL-6	ELISA	increase
Zhou *et al.*, 1993	rats	EF, PR or CAS	EF: 1 to 16 footshocks; PR: 60 min; CAS: 64 min exposure to 16 shocks	EF: after 1, 3, 8 or 16 footshocks; PR and CAS: sacrificed immediately after last stressor	plasma IL-6; IL-6 activity; IL-6 production	*IL-6 production*: splenic cell culture; *IL-6 activity*: IL-6 bioassay	increase (in plasma IL-6); decrease)in IL-6 production from spleen cells and PBMCs)
Shizuya *et al.*, 1997	rats	PR (=restraint stress)	4 h	immediately to 24 h	IL-6 mRNA and IL-6R mRNA in midbrain and hypopthalamus	RT-PCR	*Midbrain*: increase (IL-6 mRNA); decrease (IL-6R mRNA) *Hypothalamus*: no change (in IL-6 mRNA); decrease (IL-6 mRNA)
Kitamura *et al.*, 1997	mice	IMO	1 h	immediately	serum IL-6; liver IL-6 mRNA; spleen IL-6 mRNA	*serum IL-6*: *ELISA*; liver and *spleen IL-6 mRNA*: immunohisto-chemistry	increase (in serum IL-6); increase (in liver and spleen IL-6 mRNA)
Ishikawa *et al.*, 2001	rats	IMO	1 h		serum IL-6	ELISA	increase

IMO, immobilisation stress; RT-PCR , reverse transcriptase-polymerase chain reaction; EF, electric footshock; PR, physical restraint; CAS, conditioned aversive stimulus; PBMC, peripheral blood mononuclear cell; IL-6, interleukin-6

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IL-6 from monocytes, endothelial cells and fibroblasts (Waage *et al.,* 1990). Also, dexamethasone was shown to increase IL-6 receptor mRNA in a rat hepatoma cell line (Geisterfer *et al.,* 1995).

It has now been established that the HPA axis indeed exerts effects on cytokine release, which paves the way for discussing effects that cytokines have on the HPA axis in the following section.

2.5.3 Cytokine influence on the HPA axis

2.5.3.1 Hypothalamus and pituitary

Intrahypothalamic administration of IL-1ra produces a significant reduction in the plasma ACTH response to immobilization in rats, suggesting that IL-1 has a role in regulation of the HPA axis response to stress that is unrelated to the more commonly accepted role in infection or inflammation (Shintani *et al.,* 1995a; Shintani *et al.,* 1995b). In support of this, others have shown increases in the hypothalamus of (1) IL-1β mRNA expression (Minami *et al.,* 1991; Suzuki *et al.,* 1997), (2) IL-1ra mRNA (Suzuki *et al.,* 1997), and (3) IL-1 bioactivity (Shintani *et al.,* 1995b) after 30 min of immobilization stress in the rat. Also (as mentioned before), increased IL-6 mRNA was measured in the midbrain 4-24 h after restraint stress (Shizuya *et al.,* 1997). Other studies suggest that IL-1 may also mediate some behavioural effects of non-inflammatory/infectious stresses, for example, enhancement of fear conditioning (Maier and Watkins, 1995). Our interests, however, focus on the interaction that exists between cytokines and various other components of the HPA axis, e.g. GCs and GRs.

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2.5.3.2 Cytokines and adrenal glucocorticoid secretion

Recalling from section 2.2.1, GCs are secreted from the outer layered adrenal cortex cells whereas catecholamines and other neurotransmitters are secreted from the inner layered adrenal medulla. Also, large constitutive pools of IL-1α and –β have been identified in the adrenal glands: IL-1α, IL-1β and IL-1ra proteins have been detected in adrenal chromaffin cells (cells situated in the adrenal medulla and named due to positive staining with chromium salts; traditionally known to secrete the rush of adrenaline that is secreted after activation of the 'fight or flight' response) (Schultzberg *et al.,* 1989; Schultzberg *et al.,* 1995). Interleukin-1α, IL-1β, IL-1β converting enzyme (ICE) and IL-1ra mRNA have been shown to be present in the adrenal cortex and are markedly elevated in both the adrenal medulla and cortex 90 min after LPS administration (Schultzberg *et al.,* 1989; Schultzberg *et al.,* 1995; Nobel and Schultzberg, 1995; Tingsborg *et al.,* 1997). The IL-1-related cytokine, IL-18 (also known as IL-1γ), is also synthesized within the rat adrenal cortex (mainly in the zonas reticularis and fasciculata) and is strongly induced by acute cold stress (Conti *et al.,* 1997).

Rat adrenal gland extracts contain IL-6 mRNA which is markedly induced 2 h after LPS administration (Gadient *et al.,* 1995; Muramami *et al.,* 1993; Schobitz *et al.,* 1993). The zonas fasciculata and reticularis secrete small amounts of TNF-α and IL-6, but the primary source of IL-6 in the rat adrenal is the zona glomerulosa (Judd and MacLeod, 1991; Judd and MacLeod, 1992; Judd and MacLeod, 1995; Judd *et al.,* 1990). The secretion of IL-6 from adrenal glomerulosa cells is stimulated by LPS, IL-1α and IL-1β (Judd and MacLeod, 1992; Judd and MacLeod, 1995; Judd *et al.,* 1990). In addition, the secretion of IL-6 from these cells is enhanced by protein kinase C (PKC) activators, ionomycin, prostaglandin E_2, forskolin and angiotensin II (Judd and MacLeod, 1991; Judd *et al.,* 1990).

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The above data clearly indicate a relationship between these stress cytokines and the adrenal gland itself; however, it does not shed light on any possible interaction between them and GC production or secretion. Some *in vivo* studies have indicated a significant effect of exogenous cytokines on GC secretion in the adrenals, but the physiological relevance of these effects is doubted (Roh *et al.,* 1987; Andreis *et al.,* 1991; Gwosdow *et al.,* 1992; Mazzocchi *et al.,* 1993). One reason cited is that the doses required to observe direct effects of, for example, IL-1β stimulation of adrenal corticosterone secretion *in vivo* have been extremely high (35 μg/rat) (Roh *et al.,* 1987). As noted by Turnbull and Rivier, it seems unlikely that a direct action of a cytokine on the adrenal can account for the rapid *in vivo* effects of administered cytokines on plasma GC concentrations (Turnbull and Rivier, 1999). Also, an alternative role for the cytokines could be to influence GR expression, which is the topic of the next section.

2.5.3.3 Cytokine regulation of GR levels and peripheral effects

Several studies on GR have shown that *in vivo* and *in vitro* administration of pro-inflammatory cytokines modulates GR levels (i.e. influences GR resistance) (Betancur *et al.,* 1995; Dovio *et al.,* 2003; Masera *et al.,* 2000; Falus *et al.,* 1995). For the purpose of clarity, cytokine effects on GR levels and/or binding capacity are mentioned separately from and before cytokine effects on GR-mediated gene regulation are discussed.

Cytosolic GR levels were shown to be up-regulated in IL-1α-treated L929 cells in the absence of dex (Pariante *et al.* 1999). *In vitro* treatments with IL-1-inducers, endotoxin and lipopolysaccharide (LPS), or with IL-1β have also resulted in increased GR availability for ligand assessed using e.g. whole cell uptake binding method (Falus *et al.,* 1995 – CESS cells, U937 cells, HepG2 cells; Verheggen *et al.,* 1996 – BEAS S6 cells and BEAS 2B cells); whereas *in vivo* treatments

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resulted in decreased GR maximal binding capacity when radioligand binding assay was used (Liu *et al.,* 1993).

Pariante *et al.* demonstrated that 24 h treatment with IL-1α inhibits dex-induced translocation of the GR from cytoplasm to nucleus in L929 cells (Pariante *et al.* 1999). In the same cells, they also showed that 24 h pretreatment with IL-1α or 24 h coincubation with IL-1α led to significant reduction in dex-induced GR-mediated gene transcription, an effect reversed by IL-1ra. One study, in contrast, has shown that *in vitro* treatment with TNF-α has a stimulating effect on GC-induced transcriptional activity of GR (assessed using radioligand binding) in L-929 cells (Costas *et al.,* 1996). These experiments involved cells that were chemically shocked after they were transiently transfected with a MMTV-CAT plasmid (Costas *et al.,* 1996), which is a method that has been used before to measure activation of the GR (Hollenberg & Evans, 1988). Chemical shock is known to increase GR function; therefore, such manipulation confounds interpretation of the results (Sanchez *et al.,* 1994). It is, therefore, a fair conclusion that IL-1 or IL-1-inducers up-regulate GR levels and/or binding capacity after exposure to stressors other than chemical shock. On the other hand, the studies mentioned above support the concept that cytokines negatively modulate GR transactivation.

In the case of endotoxin, resistance to the inhibitory effects of Dex on corticosterone secretion has been shown (Weidenfeld and Yirmiya, 1996). *In vivo* treatment of mice and *in vitro* treatment of a hepatoma cell line with IL-1α and –β has been found to decrease GC-stimulated induction of PEPCK, the enzyme responsible for synthesis of phosphoenolpyruvate during gluconeogenesis (Hill *et al.,* 1986; Hill *et al.,* 1988; McCallum *et al.,* 1988). The degree of maximum inhibition of induction of this enzyme was comparable to the percent inhibition of GR-mediated gene transcription found in the study of Pariante *et al.* (McCallum *et al.,* 1988; Pariante *et al.,* 1999). From this it is evident that IL-1 has been

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studied more intensively than IL-6, which necessitates investigation into the role that IL-6 plays in regulation of GC action.

To my knowledge, three *in vitro* studies have investigated the direct effects of IL-6 on GR. It has been shown that IL-6 up-regulates GR in Kaposi's sarcoma, lymphoid and osteoblast-like cells (Guo *et al.,* 1996; Rakasz *et al.,* 1993; Dovio *et al.,* 2001). One group thus far focused on an *in vivo* investigation into the IL-6-GR interaction and found that IL-6 has a direct stimulatory effect on neuronal GR (in dorsal horn of spinal cord) levels in rats (Wang *et al.,* 2004). This study, focusing on a central interaction, stimulated our interested in studying the same interaction peripherally, especially after exposure to psychological stress.

Since glucocorticoid resistance has been discussed before, it is imperative to briefly consider such resistance in light of the involvement of stress in the inflammatory response.

2.5.4 Inflammation-based glucocorticoid resistance

In their publication on chronic psychological stress, Miller *et al.* proposed a *glucocorticoid-resistance* model in an attempt to explain the impact of stress on inflammatory conditions (Miller *et al.,* 2002). The basic premise of this model is that chronic stress diminishes the immune system's sensitivity to glucocorticoid hormones that normally terminate the inflammatory response (Miller *et al.,* 2002). In more detail, the notions of this model are as follows (Miller *et al.,* 2002):

a) Chronic stress elicits secretion of the hormonal products of the HPA and sympathetic adrenal medullary (SAM) axes.
b) Continued high exposure to these hormones cause white blood cells (WBC) to mount a counter-regulatory response and down-regulate the expression and/or function of receptors responsible for binding GC hormones.

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c) This receptor down-regulation diminishes the immune system's capacity to respond to cortisol's anti-inflammatory actions.

d) Depending on the extent of the receptor down-regulation, inflammatory processes may flourish and the course of the disease may subsequently worsen.

These studies provided tentative proof for several notions of the glucocorticoid-resistance model, but the authors advise against definite conclusions before future studies replicate their findings (Miller *et al.,* 2002). They also mention the importance for future studies to delineate the mechanism responsible for IL-6-induced glucocorticoid sensitivity (Miller *et al.,* 2002). Their theory also does not explain the adrenal gland's cytokine production and release functions. It instead focuses only on immune cells for this function.

It is clear from the information presented in this chapter that knowledge regarding the peripheral role and actions of IL-6 in response to psychological stress is limited. Over the past 10-15 years it has become clear that IL-1 plays a much bigger role in initiation of the stress response than IL-6. However, IL-6 may play a more important role in tissue responsiveness than initially thought. We therefore set out to design an experiment not only to investigate the function of circulating IL-6, but also to gain a better understanding of its role in metabolic tissue-responsiveness *in vivo* after exposure to mild psychological stress. Our interest is based on whether pro-inflammatory cytokines exert any direct peripheral effects on GC action *in vivo*, and the next chapter describes the methodology we employed to investigate this.

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CHAPTER 3 ANIMAL CARE, MATERIALS AND METHODS

This study was part of a collaborative effort between myself and a PhD-student in our department, Carine Smith[1], whom deserves acknowledgement for preparation of the antibody and for exposing the animals to stress and injections, as well as for supervising the daily care of the animals according to guidelines as set out by subcommittee C of the University of Stellenbosch. Smith also measured the corticosterone levels, the method of which is described in her thesis (Smith *et al.,* 2004), hence the decision to exclude it from this thesis.

3.1 Materials and equipment

The anti-IL-6-antibody was obtained from R&D systems, Germany (see paragraph 3.3). An ultra-turrax homogeniser was obtained from Janke & Kunkel KG, Germany. [1,2,4,6,7-H]Dexamethasone ([^{3}H]-Dex, specific activity: 83 Ci/mmol) was obtained from Amersham (TRK 645). The aprotinin, Coomassie Bradford Reagent, α-monothiglycerol (α-MTG), α-ketoglutaric acid (α-KG), pyridoxal-5'-phosphate (PLP), bovine serum albumin (BSA), imidazole hydrochloride, L-glutamine (Gln), hydroxylamine (NH_2OH), manganese chloride ($MnCl_2$), adenosine-5'-diphospate sodium salt (ADP) and phosphate buffered saline (PBS) were obtained from SIGMA (South Africa). The phenylmethylsulfonyl fluoride (PMSF), dextran T-70, DL-dithiothreitol (DTT), leupeptin and dexamethasone (dex) were obtained from Sigma-Aldrich, South Africa. Saarchem and Merck Laboratory Supplies (Pty) Ltd (South Africa) supplied the sucrose, ethylene diamine tetra-acetic acid disodium salt (EDTA), sodium molybdate dihydrate, L-tyrosine (Tyr), ethanol, potassium hydroxide (KOH) and hydrochloric acid (HCl). The other chemicals used were Tris(hydroxymethyl)aminomethane (GR buffer substance or Tris buffer, Merck,

[1] see Smith, 2004.

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South Africa), Tris-HCl (Merck KGaA, Germany), activated charcoal (Fluka), scintillation fluid (Zinsser Analytic), *di*-potassium hydrogen orthophosphate (K_2HPO_4, Merck, Darmstadt, Germany), ferric chloride ($FeCl_3$, NT Laboratory Supplies (Pty) Ltd, South Africa) and trichloracetic acid (TCA, Sigma Diagnostics, Inc., USA).

3.2 Experimental animals

Forty (40) male Wistar rats were used in this study because the oestrus cycle in female rats causes changes in hormonal profiles, which may confound results pertaining to corticosterone levels. Rats were housed in standard rat cages and fed rat chow and tap water *ad libitum*. Temperature in the housing facility was controlled at 21 °C and lights set to a 12-hour light/dark cycle (lights on at 7 am). Rats were bought from a nearby breeding facility (University of Cape Town), weighed and earclipped for identification, and allowed 3 weeks to acclimatize before initiation of the study protocol. During the acclimatization they were handled and weighed once per day (7 times per week) to accustom them to this procedure. No more than five (5) rats were housed together in a standard cage.

3.3 Preparation of anti-IL-6 antibody treatments

An antibody raised in goats immunised with purified *E.coli*-derived recombinant rat IL-6 (AF506, R&D Systems, Germany) was used as intervention treatment in this study. 100 ug lyophilised antibody was reconstituted with 1 ml sterile PBS, according to manufacturer's instructions, to yield an antibody concentration of 0.1 mg/ml. This stock solution was further diluted in sterile PBS to yield a final concentration of 2 ug/ml, so that an injection of 0.5 ml per day would result in a dose of 1 ug/rat/day of anti-IL-6 antibody, in accordance with the manufacturer's indicated neutralisation dosage, as well as with previous studies (Hogan *et al.,*

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2003; Arruda *et al.,* 2000). This solution was kept at 4 °C for the duration of the protocol (stable for one month at this temperature, according to manufacturer).

3.4 Intervention

For the intervention, the rats were divided into four experimental groups of 10 rats each: control placebo (CP), control antibody (CA), immobilisation placebo (IP) and immobilisation antibody (IA). Body mass measurements (taken at 08:00 am each morning) were recorded from 4 days prior to application of the first stress treatments, during the 4 days of the intervention as well as on the morning of sacrifice. Placebo rats (CP and IP) were injected intraperitoneally with 0.5 ml of sterile saline (0.9% NaCl) once per day, and antibody rats (CA and IA) were injected intraperitoneally with 0.5 ml of anti-IL-6 antibody solution (1 μg/day), once per day, prepared as described above. The injections were given 30 minutes before each bout of immobilisation stress. The stressed groups were subjected to immobilisation stress once per day, which was achieved by immobilising the rats (from 09:00 to 11:00 am) in immobilisation chambers (dimensions: 20 x 8 x 8 cm) for 2 hours. Both the injection treatment and immobilisation stress protocols lasted 4 days, after which rats were sacrificed by decapitation (24 h after last bout of immobilisation stress). After the rats were decapitated and their blood collected into standard SST tubes by drainage of the aorta (Smith, 2004), I dissected out rat skeletal muscles (gastrocnemius and vastus) and liver, which were immediately frozen in liquid nitrogen for storage at -80°C until used.

3.5 Sample preparation and assays

TAT activity and GR binding capacity was determined in the liver and vastus muscles, whereas GS activity was determined in the gastrocnemius muscle. The same vastus and liver cytosolic preparations used for determination of GR binding capacity were also used for determination of TAT activity.

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The following sub-sections describe the preparation of tissue (3.5.1.1) for use in the binding assays and the methodology for the assay itself (3.5.1.2), which will be followed by description of the method for determining levels of enzyme activities (3.5.2 and 3.5.3).

3.5.1 Radioligand binding assay to determine GR levels

3.5.1.1 Preparation of muscle and liver cytosol

Cytosol for the radioligand binding assay was prepared according to the method of Kalimi and Hubbard as modified by others (Kalimi and Hubbard, 1983; Al-Mohaisen *et al.,* 2000; Sun *et al.,* 1999). The same areas of each of the livers were thawed, weighed, cut into fine pieces and homogenised (using an Ultra-turrax homogeniser) in cytosol buffer A (10 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml aprotinin) at 4°C and at a tissue to buffer ratio of 1:2 (wt/vol), e.g. 1 g of tissue was homogenised in 2 ml of buffer. The homogenate was ultracentrifuged at 100 000 × *g* for 1 hr at 4°C. The upper fatty layer was collected with a pipette before it was discarded and the crude cytosol treated with 100 μl of dextran-coated charcoal (DCC: 3.75 g of charcoal and 0.375 g of dextran T-70 made up in 100 ml of 10 mM Tris-HCl, pH 7.5) for 15 min at 4°C to remove endogenous steroids (the DCC solution was simply added to the crude cytosol after removal of the fatty supernatant). This was followed by a centrifugation step (3 000 rpm for 10 min at 4°C). The resulting clear supernatant was referred to as 'liver cytosol' and was subsequently used for the ensuing radioligandbinding assay after a 20-fold dilution in cytosol buffer A. The protein concentration of the undiluted liver cytosol was determined with the Bradford protein determination assay (Bradford, 1976). A 5-fold dilution of each sample was made with distilled water (final volume: 60 μl), to which 250 μl of Coomassie brilliant Blue reagent was added and 5 minutes allowed for the reaction to take place. The absorbance values were determined spectrophotometrically at 595 nm, which was used to calculate the protein

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concentration within each sample by extrapolation from a standard curve plotted from known protein concentrations.

'Muscle cytosol' was prepared from vastus muscle in similar fashion to preparation of the liver cytosol, except for the following alterations:

1. Cytosol buffer A was replaced with cytosol buffer B (10 mM Tris-HCl (pH 7.5), which contained 0.25 M sucrose, 1.5 mM EDTA, 10 mM sodium molybdate, 5 mM DTT, 10 mM monothioglycerol (MTG), 2 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). During the optimization experiments, the protease inhibitors were found to interfere with GR stability in the liver cytosol, hence the use of these two different buffers, buffers A and B.
2. Both vastus muscles of each rat were pooled to measure binding affinity.
3. No dilution of muscle cytosol was necessary to determine binding capacity.

3.5.1.2 Radioligand binding assay

To determine GR binding capacity, 250 μl cytosol (20-times diluted liver or undiluted muscle) was incubated with 10 μl of 400 nM [^{3}H]dexamethasone (made up in ethanol) in the presence (non-specific binding) or absence (total binding) of 10 μl of 1 000-fold excess of unlabelled dexamethasone (0.4 mM made up in ethanol), in 130 μl cytosol buffer (A for liver and B for muscle), respectively, for 24h at 4°C (Al-Mohaisen *et al.,* 2000). In the absence of unlabelled dexamethasone, 10 μl of ethanol was added instead to control for volume in total binding samples. To separate free and GR-bound dexamethasone 100 μl of DCC suspension (as described above in section 3.5.5.1) was added for 10 minutes at 4ºC (Beato and Feigelson, 1972). The DCC-containing sample was centrifuged at 3 000 × *g* for 10 min, at 4°C. This step was followed by collecting 350 μl of supernatant and adding it to 3 ml scintillation fluid, in which radioactivity was measured in a Beckmann LS 3801 scintillation counter. Control samples were included in the experiment to check for counting efficiency (CE, the ratio between

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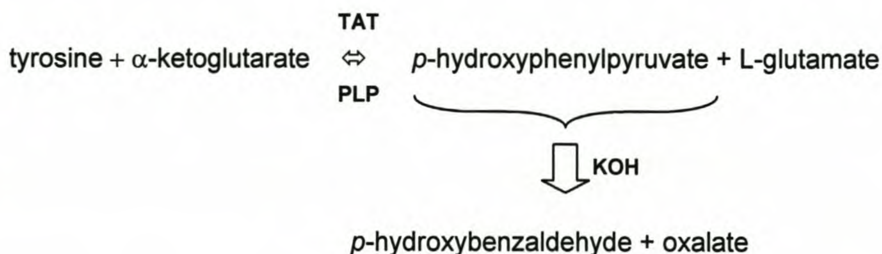
the number of particles or photons counted with a radiation counter and the number of particles or photons of the same type and energy emitted by the radiation source), ligand depletion, total radioactivity added and the ability of DCC to remove radioactive steroid. The DCC treatment (on average) was 95% effective; CE was calculated as 35% and ligand depletion was less than 10%. Specific receptor binding (SB) was determined as the difference between total receptor binding (TB) and non-specific receptor binding (NSB). The radioactive counter expressed radioactivity levels as numeric values in counts per minute (cpm), which in turn was converted to units expressed as fmol/mg (femtomol per milligram) protein. Examples of calculations are also shown in Appendix A.

3.5.2 Tyrosine aminotransferase assay

The cytosol used for GR-binding (section 3.5.1.1) could also be used to determine TAT activity in both skeletal muscle and liver. The TAT assay used in this study is a modification of that described in various publications (Diamondstone, 1966; Granner and Tomkins, 1970a; Schäcke *et al.,* 2004). TAT converts tyrosine to *p*-hydroxyphenylpyruvate (pHPP), in the presence of α-ketoglutarate. The TAT assay depends on the alkali-catalyzed conversion (by adding KOH) of the reaction product, pHPP, to *p*-hydroxybenzaldehyde (pHBA) and oxalate. This reaction is indicated below and the assay explained further:



The absorbance of pHBA was measured at 330 nm.

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The TAT-reaction mixture for one sample consisted of (i) 0.8 ml of 7 mM tyrosine, made up in 125 mM K_2HPO_4 buffer at pH 6.8, (ii) 20 μl of 500 mM α-ketoglutarate (α-KG) made up in dH_2O, and (iii) 10 μl of 5 mM pyridoxal-5'-phosphate (PLP) made up in dH_2O. The liver cytosol samples were diluted a 100-fold in the K_2HPO_4 buffer containing 5 mg/ml bovine serum albumin (BSA), 1 mM EDTA and 1 mM dithiothreitol (DTT). The muscle samples were not diluted for TAT activity determination. After the tyrosine (800 μl), PLP (10 μl) and α-KG (20 μl) were added to 4 microcentrifuge tubes ('eppies'), 100 μl of the cytosol (liver or muscle) was added to 3 eppis. Three (3) eppies were placed in a 37°C waterbath for 10, 20 and 30 minutes, respectively. After the indicated amounts of time, 70 μl of 10 N KOH (potassium hydroxide) was added to these three eppies to stop the reaction in which tyrosine was converted to pHPP. Spectrophotometric analysis against a blank sample was performed 30 minutes after the KOH had been added, to allow for conversion of pHPP to pHBA. The blank sample was made by adding the KOH to the reaction mixture *before* the addition of cytosol to the fourth eppi. TAT activity was calculated by using a molar extinction coefficient of 19 900 $M^{-1}cm^{-1}$ for pHPP (Diamondstone, 1966). One unit of enzyme activity is the quantity that catalyzes the formation of 1 μmole of pHPP per minute at 37°C. Calculations are shown in Appendix A. Results are expressed as units per milligram of protein per hour (U/mg prot/h).

3.5.3 Glutamine synthetase

The method of GS activity determination was performed in the rat gastrocnemius muscle according to the methodology of Meister, as modified by others (Meister, 1985; Minet *et al.,* 1997; Machado *et al.,* 2001), which measures the formation of γ-glutamyl hydroxamate from glutamine, as illustrated in the reaction scheme below:

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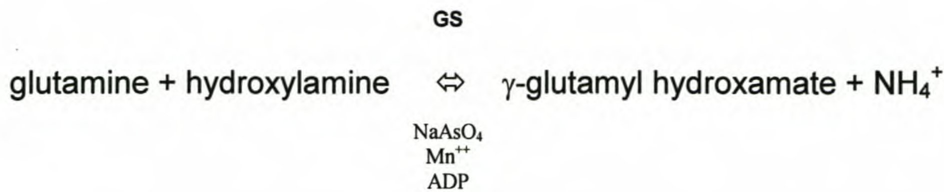

3.5.3.1 Sample preparation

The muscle was thawed and placed in 9 volumes of 50 mM imidazole, pH 6.8, at 4°C. The tissue was cut into finer pieces with a pair of scissors before it was homogenized with the ultra-turrax. The homogenate was centrifuged at 4 500 × *g* for 15 min, at 4°C, and the supernatant was used for the enzyme assay. Protein determination was performed by using the Bradford Protein Determination Method (Bradford, 1976), as described in section 3.5.1.1.

3.5.3.2 GS assay

For determination of GS enzyme activity, the following reaction mixture was prepared in two 5 ml tubes (one tube serving as blank): 200 μl of 250 mM imidazole chloride, pH 6.8; 100 μl of 500 mM L-glutamine (L-Gln) made up in 50 mM imidazole, pH 6.8; 200 μl of 125 mM hydroxylamine (NH_2OH) made up in dH_2O; 100 μl pf 250 mM sodium arsenate (Na_2HAsO_4) and 200 μl of 10 mM manganese chloride ($MnCl_2$) (both solutions made up in dH_2O). The cytosol (100 μl) and 100 μl of 1.6 mM freshly prepared ADP (in this order; ADP made up in dH_2O) were added to the active tube just before it is placed in a 37°C water bath for 25 minutes (the blank sample is incubated at 37°C as well). No ADP is added to the blank tube (thus, the blank contains the total incubation mixture except for the ADP - dH_2O is used to fill up this volume). The reaction is stopped by addition of 2 ml stop solution (2.42% $FeCl_3$ and 1.45% trichloracetic acid in 1.82% HCl). Insoluble material is removed by centrifugation (4 500 × *g* for 5 min) and the absorbance of the supernatant was measured at 540 nm to quantify the formation of γ-glutamyl hydroxamate, for which a molar extinction coefficient of

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1.08 $M^{-1}cm^{-1}$ was used (Montanini *et al.,* 2003). GS activity is expressed as nmol/min/g prot (nanomoles per minute per gram protein). Calculations are shown in Appendix A.

3.6 Statistical analysis

Data were analysed using GraphPad Prism (version 4.00, GraphPad Software, San Diego, California, USA). The values were calculated as means ± SEM for each data set. For analysis of the main effects of either immobilisation stress or anti-IL-6-antibody injections, data were analysed by a two-way ANOVA and Fischer's post-hoc test. To determine whether any interaction occurred between the two experimental factors (antibody *vs.* stress), the same statistical method described above was used. Interaction indicated that the effects of these two factors were significantly additive or counteractive. Correlation analyses were performed using Pearson's correlation coefficient. For all the data, *p*-values at least <0.05 were considered significant.

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CHAPTER 4 RESULTS AND DISCUSSION

Psychological stress has been shown to induce the secretion of glucocorticoids (GCs) and cytokines (Turnbull and Rivier, 1999). Current paradigms include a bidirectional communication between GCs and cytokines (Maier, 2003), however, few studies have investigated the blocking of a particular cytokine and the subsequent effects on the physiological response to stress. In addition, few studies have investigated the effects of cytokines on the tissue responsiveness to GCs.

In the current study we sought to investigate the effect of blocking the action of IL-6, by means of an anti-IL-6-antibody injection, on the stress response elicited in Wistar rats by a mild chronic psychological stressor. We specifically chose to investigate the effects of IL-6 because it has been shown that mild psychological stress in rats induces the production of IL-6 (LeMay *et al.,* 1990; Zhou *et al.,* 1993). In the study by Zhou *et al,* the elevation in plasma IL-6 occurred despite no sign of endotoxin, tissue damage or inflammation, all of these being conditions which are expected to increase IL-6 levels (Zhou *et al.,* 1993). The actions of IL-6 have been investigated before by using an anti-IL-6-antibody (IL-6-Ab) in an osteoblast cell culture system (Masera *et al.,* 2000), which was one of only a few studies which studied the effects of a cytokine on tissue responses to GCs. Also, this study showed specific interest in GR levels, and since we were also interested in the expression of GR after exposure to psychological stress, we decided to employ the IL-6-Ab approach *in vivo*. Other *in vivo* studies on the interactions between IL-6 and the HPA axis have all used the approach of knockout-studies in mice (Manfredi *et al.,* 1998; Turnbull *et al.,* 2003). Although that approach is valuable, the effect of neutralising the cytokine is not limited only to the time period of interest, which further motivated the use of the anti-IL-6-Ab in this study.

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To elicit a mild chronic psychological stress, we chose an intervention that consisted of a brief immobilisation (2 hours) each day for 4 consecutive days. Previous studies have shown that 3 days of mild psychological stress (2-3 hours/day) is sufficient to induce significant loss of body mass in stressed animals (Harris *et al.,* 1998; Harris *et al.,* 2002; Hu *et al.,* 2000; Rybkin *et al.,* 1997), hence our decision to apply immobilisation stress for 2 h each day. Also, it was shown that the peak level of serum corticosterone was reached within 60 min of immobilisation stress and lasted as long as the experimental duration (Hu *et al.,* 2000). We applied stress between between 9 AM and 11 AM. This specific time period was chosen because (1) it prevents a confounding influence of circadian variation in plasma corticosterone levels from day to day, and (2) plasma corticosterone levels are lowest in the morning (Hu *et al.,* 2000). Specifically, as indicated in Chapter 3, 40 rats were divided into 4 experimental groups (n=10 for each group): CP, control placebo; CA, control antibody; IP, immobilised placebo; IA, immobilised antibody. The immobilised groups were returned to their normal housing after each bout of immobilisation stress. The antibody groups received anti-IL-6-antibody injections, which were administered intraperitoneally.

As evident by the cited literature in Chapter 2, much work has been done that confirms GC-based inhibition of cytokine action, particularly in the brain. Also, the effect of cytokine action on the HPA axis has been investigated intensively. However, the physiological implications of increased IL-6 levels after stress remain largely unknown, especially at the level of metabolic tissue responsiveness to GCs. Due to the proven relationships between certain parameters and the stress response in rats, we chose to measure the following as indicators of stress: (i) body mass change (Harris *et al.,* 2004), (ii) serum corticosterone concentrations (Harris *et al.,* 2002), (iii) GR binding capacity (e.g. Al-Mohaisen *et al.,* 2000; Hu *et al.,* 2000) and (iv) enzymatic activities of TAT (Al-Mohaisen *et al.,* 2000) and GS (e.g. Max, 1990; Max *et al.,* 1988). Measurements for (iii) and (iv) were performed in liver and muscle tissue because these are both

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organs that have been shown to contain both GR and TAT activities (Al-Mohaisen *et al.,* 2000). In fact, most of the *in vivo* work relating to GR and TAT has been done in muscle and/or liver and therefore other types of tissue were not considered. For example, adipose tissue plays a central role in energy and fuel metabolism, but the scope of this investigation does not permit a discussion on the role of adipose tissue in metabolism. GS has been shown to exhibit GC-induced activity in the muscle (Labow *et al.,* 1999), hence our decision to measure GS in skeletal muscle. In context of this thesis, two specific amino acids are of interest: (1) glutamine is produced from glutamate (catalysed by GS) and is released from the muscle; (2) tyrosine (especially in the liver) is catabolised to fumarate and acetoacetate, which form substrates for gluconeogenesis (Chapter 2). Figure 4.1 illustrates the pathways in which these amino acids feature. What follows is the presentation of results obtained during the course of the intervention described above.

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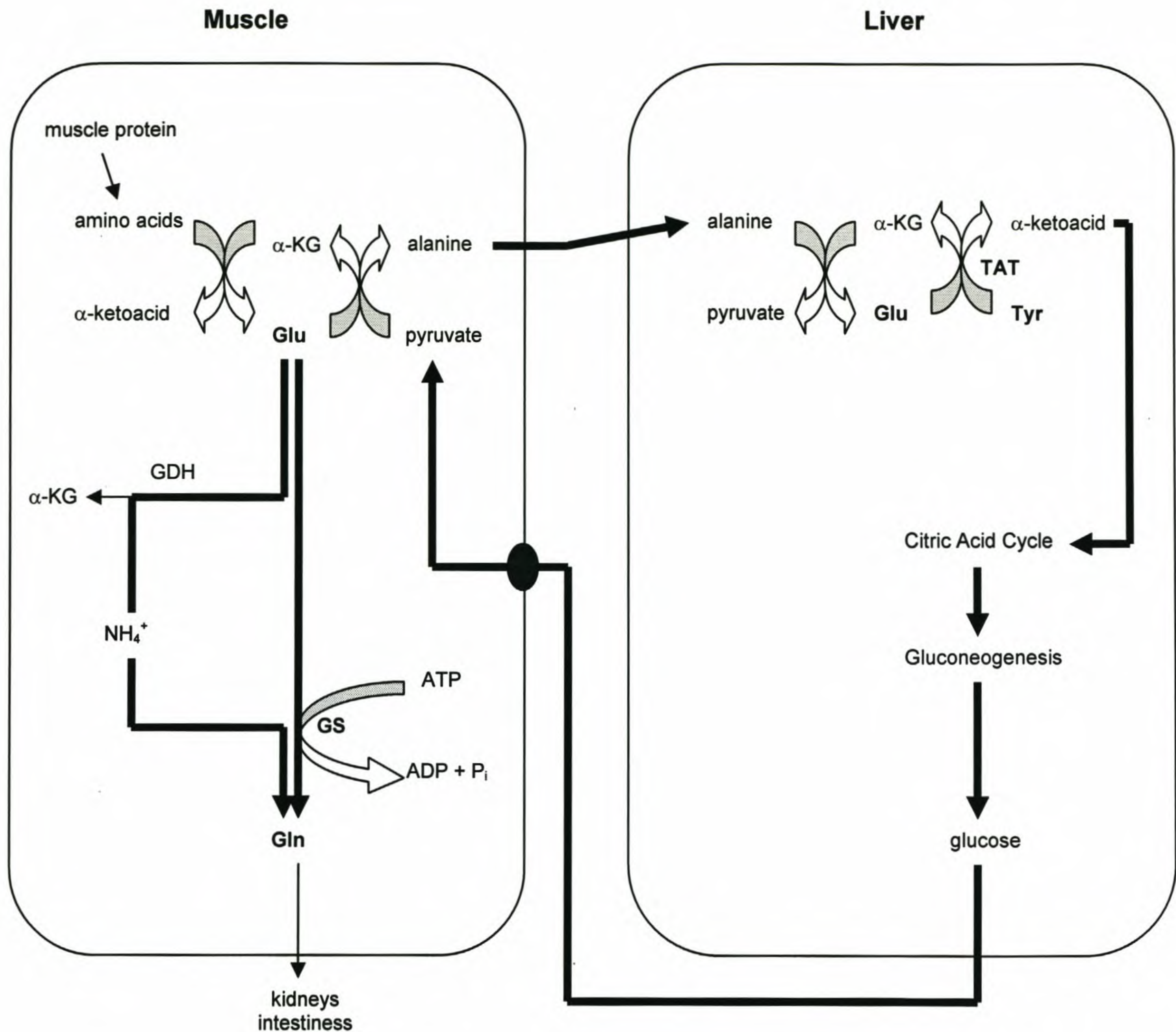

Figure 4.1 A simplified illustration of the metabolic pathways in which glutamine synthetase (GS) and tyrosine aminotransferase (TAT) are involved. During stress, muscle protein is catabolised to amino acids. GS catalyzes the reaction in which glutamine (Gln) is formed from glutamate (Glu). The Gln is used by the intestines and kidneys (acid/base regulation). TAT catalyzes the reaction in which tyrosine is metabolised to 4-hydroxyphenylpyruvate (not shown), which is ultimately catabolised to acetoacetate (oxidized by extrahepatic tissues) and fumarate (converted to oxaloacetate which is fed into the citric acid cycle). Adapted from Salway, 1994. Abbreviations: a-KG, alpha-ketoglutarate; NH_4^+, ammonium; Gln, glutamine; Glu, glutamate; AD(T)P, adenosine di(tri)-phosphate; P_i, inorganic phosphate; GDH, glutamate dehydrogenase.

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4.1 Body mass and corticosterone levels

It is known that chronic stress generally results in a decrease in body mass (Hu *et al.,* 2000; Alexandrova & Farkas, 1992) and an increase in corticosterone concentrations, despite the use of diverse stressors for varying durations (Münck *et al.,* 1984; Herman *et al.,* 1997; Sapolsky *et al.,* 1984).

The body mass of each rat was measured every day at 8 AM (1 h before the stress regime) on the 4 days prior to the start of the stress intervention, and on the 4 days during the intervention as well as on the morning of sacrifice, which was 24 hours after the last bout of immobilisation stress. Results are expressed as the average percentage change in body mass of each group (± standard error of the mean, S.E.M.) over two periods. The 'before' period includes the masses obtained before the intervention (4 days) while the 'after' period includes the masses obtained during the intervention (4 days) plus 24 hours after the final bout of stress (Fig 4.2A). We chose this method of presentation since the rats were still slowly gaining body mass in the 'before' period.

As expected, the immobilised rats lost a significant percentage of body mass when compared to controls (main effect of immobilisation - post hoc comparisons are presented in Fig 4.2A; CP vs IP, $P<0.01$; CA vs IA, $P<0.001$). This is in agreement with reports by other authors, confirming the notion that chronic stress results in a decrease of body mass (Hu *et al.,* 2000; Alexandra and Farkas, 1992). Although the protocol of this study allowed only 4 days of mild psychological stress, the stress was severe enough to induce significant loss (3-4%) of body mass in the Wistar rats. This correlates well with a report by Harris *et al.*, which indicated a 5-10% loss of body mass in Sprague-Dawley rats after exposure to restraint stress for 3 hours per day over 3 days (Harris *et al.*, 1998). Restraint stress is more severe than immobilisation stress and the protocol lasted an hour longer than ours, thus accounting for the greater body mass loss in those amimals.

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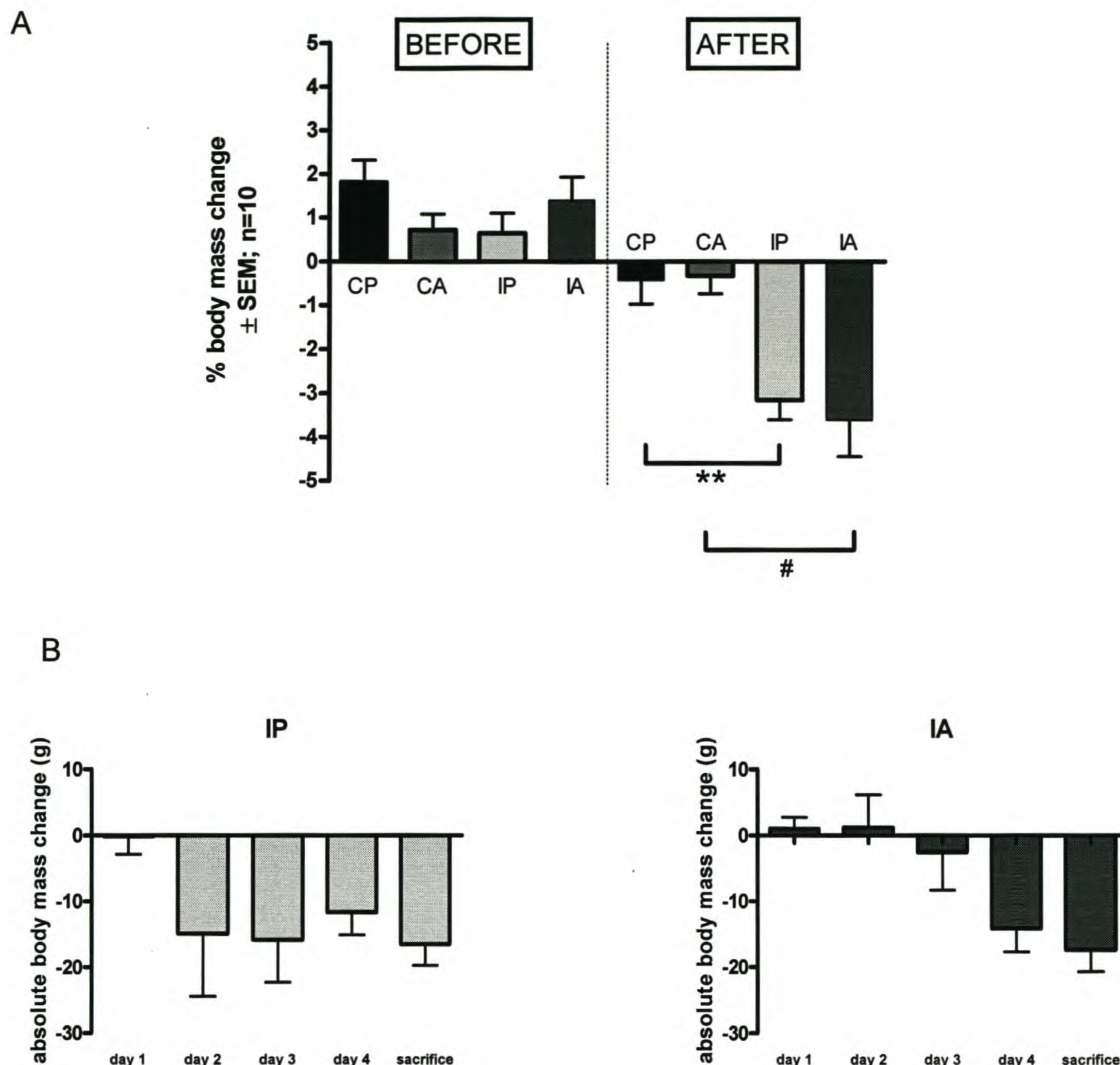

Figure 4.2. The effect of immobilisation stress and anti-IL-6-antibody treatment on the change in body mass of Wistar rats. (A) The average percentage of body mass change in the 4 different experimental groups used. The percentage change in body mass of male Wistar rats was averaged for 4 days prior to the start of the intervention protocol (BEFORE) and during the 4 days of the intervention protocol plus the day of sacrifice (AFTER). Prior to day 1 of the intervention, no difference in body mass were found between the different groups. After the intervention, the stressed groups lost weight significantly when compared to controls. **(B)** Absolute body mass change per day in the two groups of stressed rats (IP and IA). Despite no detectable statistical difference, it seems that group IP lost weight much faster than groups IA, which is reflected on the sizes of the bars on days 2-3 of the intervention. All results are expressed as means ± SEM and statistics were performed using 2-way ANOVA with Fischer post test analysis *(**P<0.01; #P<0.001)*. Abbreviations: CP, Control Placebo; CA, Control Antibody; IP, Immobilisation Placebo; IA, Immobilisation Antibody.

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Both repeated restraint stress (Harris *et al.,* 2002) and chronic overproduction of IL-6 in transgenic mice (Tsujinaka *et al.,* 1995) have been shown to independently cause muscle mass loss and reduction in body mass of rats. In our case, blocking the action of IL-6 by anti-IL-6 antibody treatment had no significant effect on the percentage change in body mass between the placebo-treated and antibody-treated rats in the control groups (no main effect of antibody and no post hoc differences - Fig 4.2A; CP vs CA). Similarly, the antibody treatment had no significant effect on the change in percentage body mass of the stressed group (Fig 4.2A; IP vs IA), both of which lost a similar amount of body mass over the course of the intervention.

Closer inspection, however, revealed that, although groups IP and IA showed no difference in accumulated body mass loss during and after the intervention, the time frame of the body mass loss might have been influenced by the antibody injection (Fig 4.2B). When the daily loss of body mass is shown, group IA does not show the same amount of weight loss on days 2 and 3 when compared to groups IP. If the antibody treatment did not cause a significant effect in the accumulated body mass loss between these stressed groups, it seemed to affect the time frame of body mass loss in those groups (Fig 4.2B, compare body mass loss of groups IP *vs.* IA, days 2 and 3). However, no significant effect could be proved with statistical analysis, which is probably due to large variations in absolute body mass loss between rats in the different groups.

If the mechanism of stress-induced body mass loss was reliant on the production of excess IL-6, we would have expected to see a lesser change in body mass in the IA group compared to the IP group. Instead, the mean accumulated body mass change for the stressed group IA (-3.61±0.83%) was slightly higher (but not significantly so) than for the stressed placebo group (IP, -3.16±0.44%). However, the antibody treatment did retard body mass loss in group IA (Fig 4.2B). This suggests that IL-6 plays a role in body mass loss especially during the earlier response (days 2-3) to a chronic stress. The fact that the IA group did lose body

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mass by day 4 could be due to a slower acting mechanism separate from IL-6, or it is possible that the rats began to produce excess IL-6 that was no longer neutralised by the antibody.

The corticosterone concentrations of the rats in the different groups were measured 24 hours after the last bout of immobilisation stress and are shown in Figure 4.3. Immobilisation stress caused a significant 2-fold up-regulation of corticosterone concentrations in the serum of rats in the stressed placebo group (main affect of stress with one significant post hoc test result - Fig 4.3: CP vs IP, $P<0.01$), agreeing with results presented by Al-Mohaisen *et al.*, who showed that immobilisation in rats caused a 2- to 3-fold increase in serum corticosterone concentrations (Al-Mohaisen *et al.*, 2000). Although there was a significant main effect of immobilisation stress on corticosterone concentration, there was also an interaction effect with the antibody resulting in no significant effect of stress as measured in the serum of rats of group IA compared to group CA. It was previously reported that IL-6 causes up-regulation of corticosterone concentrations via influences at different levels of the HPA axis, namely the hypothalamus, pituitary and adrenal glands (Navarra *et al.,* 1991; Zhou *et al.,* 1993; Muramani *et al.,* 1993; Schobitz *et al.,* 1993; Spangelo *et al.,* 1990). Our results support a role for IL-6 in increasing corticosterone concentrations of rats. Although the increase of corticosterone levels in group IA does not reach significance, it shows a slight increase when compared to the non-stressed groups (CA and CP). When taken into account that this slight increase is in the absence of IL-6, we thus speculate that IL-6 may be required for a full-blown stress response.

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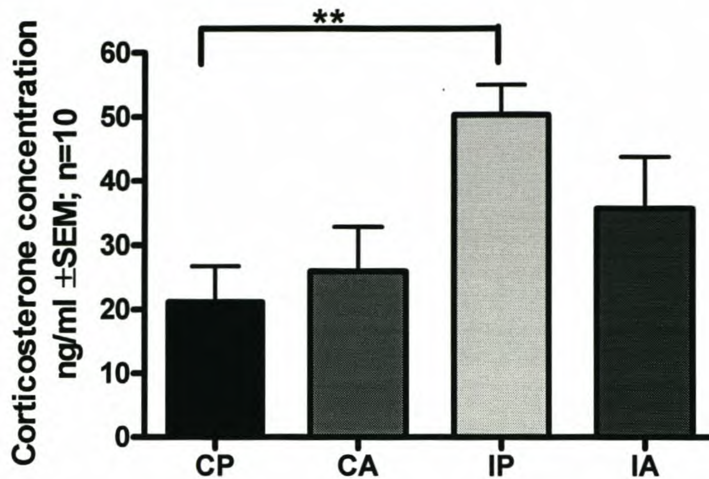

Figure 4.3 The effect of immobilisation stress and anti-IL-6-antibody treatment on the serum corticosterone levels of male Wistar rats. Serum corticosterone concentrations in control and stressed groups of the Wistar rats were measured by enzyme-linked immunosorbent assay (ELISA) kits. Results are expressed as means ± SEM and were analysed using two-way ANOVA with Fischer post tests (**$P<0.01$*). Abbreviations: CP, Control Placebo; CA, Control Antibody; IP, Immobilisation Placebo; IA, Immobilisation Antibody.

In summary, our results (showing that the immobilisation protocol decreased body mass and increased corticosterone levels of male Wistar rats) indicate that our intervention was indeed eliciting a stress response and that IL-6 may be required for an early and a full-blown activation of the stress response, certainly as measured by corticosterone levels.

4.2 Glucocorticoid receptor binding

Glucocorticoid action is mediated via its receptor in the cytoplasm of responsive cells and the magnitude (maximal response) and potency (EC_{50}) of the response is dependent on receptor levels (Simons & Thompson, 1982). Several authors have addressed the issue of studying the effects of stress on GR levels, but

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results are conflicting (see Al-Mohaisen *et al.,* 2000 for discussion on these conflicting reports). Thus we decided to measure GR levels in the liver and muscle of our experimental groups of rats. Tissue was harvested at the same time as blood samples from which the corticosterone concentrations were measured (24 hours after the last bout of immobilisation stress). The liver and muscle were chosen because the two metabolic enzymes we studied, GS and TAT, function principally in these organs during stress (Labow *et al.,* 1999; Minet *et al.,* 1997; Rosen and Milholland, 1963; Rosenberg and Litwack, 1970). To determine the levels of GR in rat liver and muscle we measured the extent of agonist (dexamethasone) binding, using radioligand binding assays. The results are presented in figures 4.4A (muscle) and 4.4B (liver).

The GR binding capacity in group CP of both liver (36.36±2.53 fmol/mg protein) and muscle (6.41±0.45 fmol/mg protein) are lower than values reported by other authors (Al-Mohaisen *et al.,* 2000; Sun *et al.,* 2002). A possible explanation involves handling of the animals. All the rats were given injections (either placebo or IL-6-Ab), which might have served as a stressor in itself and therefore could have led to down-regulation of GR levels. Handling of rats, in general, was shown to be perceived by the rodents as stressful (Gadek-Michalska & Bugajski, 2003), which leads to prolonged feedback inhibition of HPA axis activity even before the start of experimental interventions. Alternatively, other studies used different strains (e.g. Sprague-Dawley, Fischer 344 or Lewis rats *vs.* our Wistar rats), which have considerable differences in glucocorticoid responses to stress (Dhabhar *et al.,* 1993).

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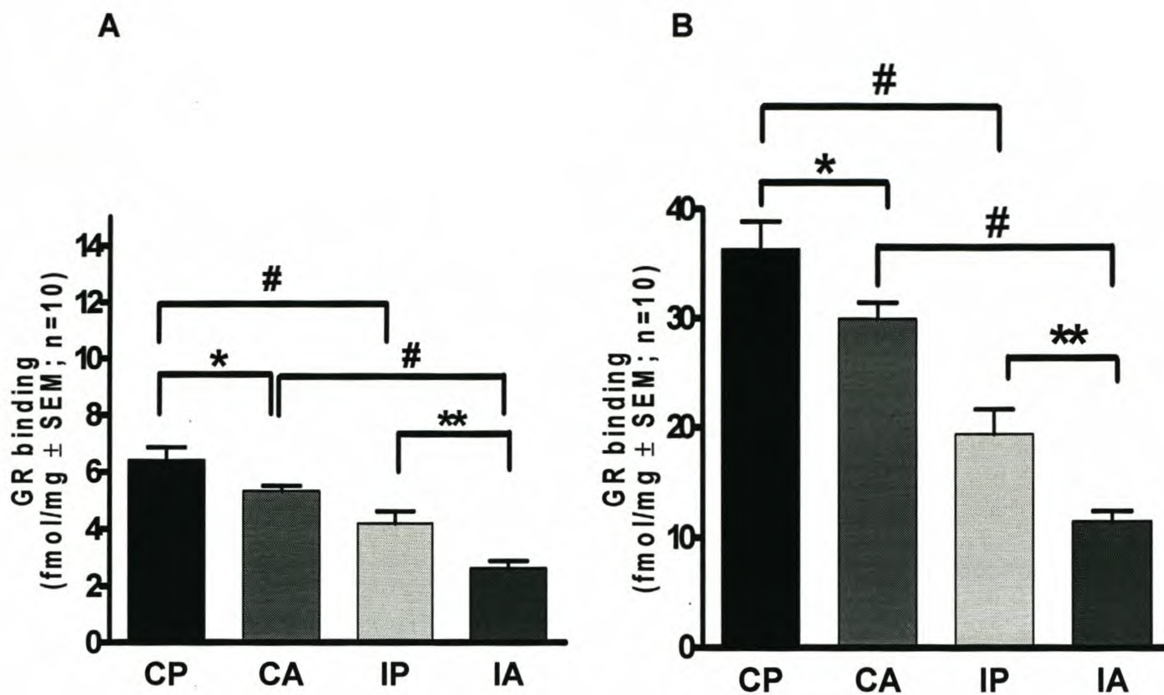

Figure 4.4 The effect of immobilisation stress and anti-IL-6-antibody treatment on the GR levels in (*A*) skeletal muscle and (*B*) liver of Wistar rats. GR levels were determined by dexamethasone binding capacity as measured by radioligand binding assay. Both stress and the anti-IL-6-antibody significantly down-regulated the GR levels. Results are expressed as means ± SEM. Statistical analysis: Two-way ANOVA with Fischer's LSD as post test. *(*P<0.05; **P<0.01; #P<0.001)*. Abbreviations: CP, Control Placebo; CA, Control Antibody; IP, Immobilisation Placebo; IA, Immobilisation Antibody.

Immobilisation stress caused a significant decrease in skeletal muscle and liver GR binding capacity (main effect of stress with post hoc results presented in Fig 4.4A and 4.4B; CP *vs.* IP, P<0.0001; CA *vs.* IA, P<0.0001). Many authors (e.g. Omrani, 1980; Sapolsky 1984; Shirwany, 1986) have previously presented similar results (decrease in GR binding capacity), whereas others have reported increases in GR binding capacity and amount of GR in studies where the effect of chronic stress was investigated (Hu *et al.,* 2000; Al-Mohaisen *et al.,* 2000; Sun *et al.,* 2002). In both the liver and muscle tissue, we measured between 17-70% decreases in GR levels between groups CP and all the other experimental groups. This correlates with previous reports that indicated decreases in GR

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levels of between 25-50% (Sapolsky *et al.,* 1984 – brain tissue; Omrani *et al.,* 1980 – hepatic tissue; Shirwany *et al.,* 1986 – hepatic tissue).

Discrepancies in results between studies have been attributed to factors such as use of different techniques and animals, duration and intensity of stress and different types of tissues under investigation. The receptor assay we used measured the total and non-specific binding capacity of the receptors in the cytosol extracts and therefore we could calculate the specific binding capacity of the receptors to dexamethasone. Two reports that indicated increases in binding activity of GRs differ from ours in the following respects: (1) we investigated the effects of a mild chronic stress whereas Sun *et al.* (Sun *et al.,* 2002) studied the effect of sepsis (a more acute and extreme stress) on muscle GR expression and binding; and (2) Al-Mohaisen *et al.* (Al-Mohaisen *et al.,* 2000) used a new approach to measuring GR in liver tissue that could not be repeated in our laboratory (despite several attempts), which makes it difficult to interpret their results in comparison to our own.

The antibody treatment caused a significant down-regulation of GR binding capacity in both skeletal muscle and liver, under basal (main effect of antibody treatment with post hoc results presented in Fig 4.4A and Fig 4.4B: CP *vs.* CA, $p<0.05$) and stressed conditions (main effect of stress with post hoc results presented in Fig 4.4A and Fig 4.4B: IP *vs.* IA, $p<0.01$). It has been shown that IL-6-antibody administration significantly decreased GR number *in vitro* in osteoblast cell lines, abolishing the up-regulatory effect of IL-6 (Masera *et al.,* 2000; Dovio *et al.*, 2001). The results of our study are the first to demonstrate this effect *in vivo* and to support the *in vitro* findings of Masera *et al.* (2000) and Dovio *et al.* (2001). Further supportive evidence was provided by authors who did not use anti-IL-6-antibodies in their studies, but who showed that IL-6 up-regulates GR in both Kaposi's sarcoma and lymphoid cells (Guo *et al.,* 1996; Rakasz *et al.,* 1993).

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4.3 Enzymatic activities

In Chapter 1 it was mentioned that, during stress, GCs induce the expression of many enzymes, basically to promote the mobilisation of amino acids (from protein degradation in the muscle) for the purpose of serving as energy substrates for other cells and for gluconeogenesis in the liver. Such GC-responsive enzymes, whose genes contain GREs, are typically found in GC target tissues and are used to indicate enzymatic responses to stress. The two enzymes we chose to study were tyrosine aminotransferase (TAT) and glutamine synthetase (GS). Traditionally, TAT activity is measured in the liver (Kenney, 1959; Rosenberg and Litwack, 1970), whereas substantial GS activity can be measured in skeletal muscle and lungs (Labow *et al.,* 1999; Abcouwer, 1995).

4.3.1 Tyrosine aminotransferase

TAT activity was determined in skeletal muscle and liver by measuring the absorbance of a reaction mixture containing *p*-hydroxybenzaldehye (pHBA), which was formed after the alkali-catalysed conversion of *p*-hydroxyphenylpyruvate (pHPP) (Diamondstone, 1966; see Chapter 3). Results are shown in Fig 4.5.

In skeletal muscle and liver, the immobilisation stress caused a significant up-regulation of TAT activity in both the placebo-treated (main effect of stress with post hoc results presented in Fig 4.5A (CP vs IP, $p<0.001$) and Fig 4.5B (CP vs IP, $p<0.0001$)) and antibody-treated rats (main effect of antibody treatment with post hoc results presented in Fig 4.5A (CA vs IA, $p<0.001$) and Fig 4.5B (CA vs IA, $p<0.0001$)). The different percentage increases of TAT activity are shown in Table 4.1.

The increases in TAT activity are in agreement with reports that also indicate increases in hepatic TAT activity after GC treatment (DuBois *et al.,* 1995;

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Ramakrishnan *et al.,* 2002) and immobilisation stress (Al-Mohaisen *et al.,* 2000). In our case, however, a higher fold-induction was measured (Table 4.1), especially in the liver after immobilisation stress. Al-Mohaisen *et al.* reported a 1.6-fold increase in hepatic TAT activity after immobilisation stress (Al-Mohaisen *et al.,* 2000), whereas we measured 2.7- and 2.6-fold inductions in the stressed groups. In the other reports mentioned, 7- and 6-fold inductions of TAT activity were reported following hormone administration (Ramakrishnan *et al.,* 2002; DuBois *et al.,* 1995; Chesnokov *et al.,* 1990).

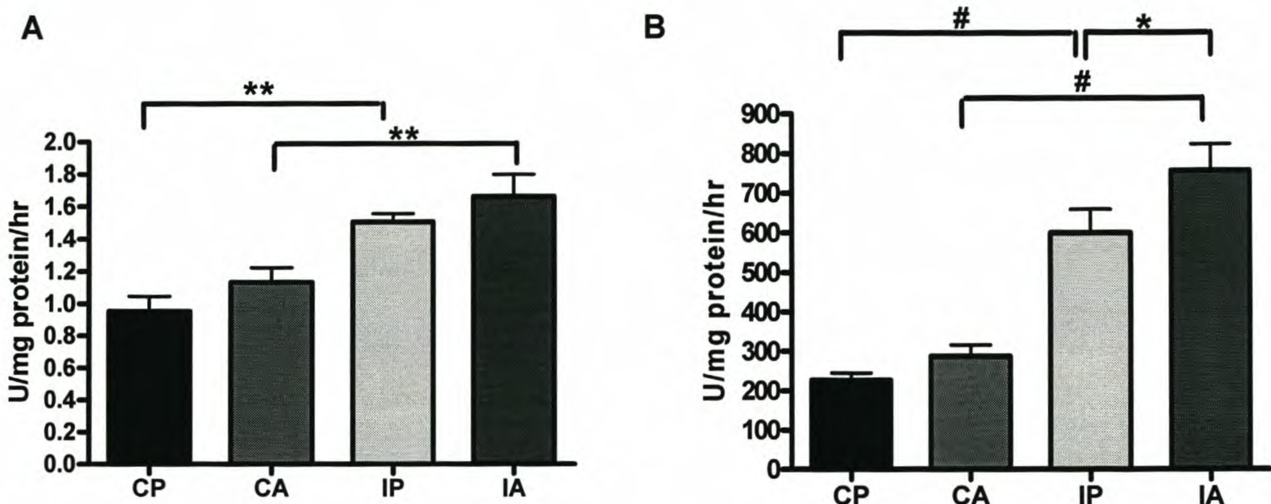

Figure 4.5 The effect of immobilisation stress and anti-IL-6-antibody treatment on the TAT activity in (A) skeletal muscle and (B) liver of Wistar rats. TAT activity was determined by following the non-enzymatic conversion of *p*-hydroxyphenylpyruvate to *p*-hydroxybenzaldehyde. Results are expressed as means ± SEM, n = 39. Statistical analysis: Two-way ANOVA with Fischer's LSD as post test. *(*P<0.05; **P<0.01; #P<0.001).* Abbreviations: CP, Control Placebo; CA, Control Antibody; IP, Immobilisation Placebo; IA, Immobilisation Antibody.

However, treatment with exogenous GCs can be considered a more extreme intervention. More importantly, we show an effect after only 2 h/day of mild psychological stress. Of added significance is the fact that we found changes in TAT activity in skeletal muscle after psychological stress, since most previous research on TAT has investigated only the liver. In our case, there is a large

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difference in the amount of TAT activity measured between these two different tissues, which agrees with a previous study performed in mink (Prieur *et al.*, 2001). We suspect that the traditional focus (with regards to TAT activity) may have been placed on the liver due to the abundance of the TAT protein in this organ, but it does not justify why studies on TAT activity in other tissues have been neglected to the extent that is has been.

TABLE 4.1. *Percentage and fold increases in TAT activity in skeletal muscle and liver of Wistar rats after exposure to immobilisation stress and anti-IL-6-antibody treatment.*

	Stressed groups		Antibody groups	
	IP vs CP	IA vs CA	CA vs CP	IA vs IP
Muscle	+ 58 %	+ 47 %	+ 19 %	+ 11 %
Fold-induction	1.58	1.47	1.19	1.11
Liver	+ 165 %	+ 159 %	+ 27 %	+ 24 %
Fold-induction	2.65	2.59	1.27	1.24

Footnote: % increase: calculation e.g. ((IP/CP)*100) - 100; fold-induction: calculation e.g. IP/CP

The effect of the antibody treatment on TAT activity was less pronounced although the main effect was significant in liver ($p<0.05$) and tending toward significance in muscle ($p<0.08$). Post hoc analysis revealed that, in both the liver and muscle tissue, there were no statistical differences between the CP and CA groups. In the muscle, no statistical difference was measured between the IP and IA groups ($p=0.25$), however, TAT activity was different between these groups ($p<0.05$) in the liver. The latter result (in the liver) indicates, therefore, that the antibody had increased TAT activity independent of stress and that IL-6 may thus inhibit TAT activity *in vivo*. In support of our results, it has been shown that IL-6c (a recombinant IL-6 molecule) strongly reduced, in a dose-dependent manner, expression of TAT in rat fetal hepatocyte cells (Zvibel *et al.*, 2004). With the exception of this last-mentioned study, there is a general lack of *in vitro* data with

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regards to enzyme-responsiveness to IL-6. Our results are the first to show, *in vivo,* that a direct relationship exists between IL-6 and TAT activities in adult rat livers. More research, therefore, is necessary to establish the exact mechanisms involved.

4.3.2 Glutamine synthetase

GS activity was determined in gastrocnemius muscle by measuring the formation of γ-glutamylhydroxamate in a reaction whereby glutamine is used as a substrate, together with ADP and hydroxylamine (Minet *et al.,* 1997; see Chapter 3). Results are shown in Fig 4.6.

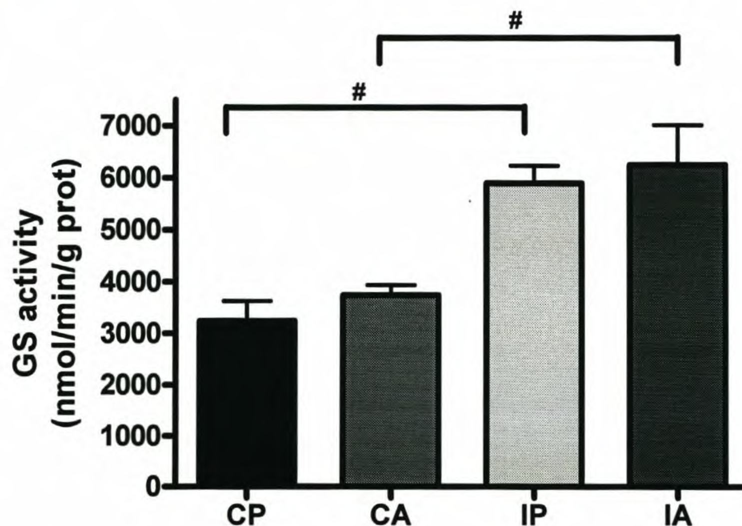

Figure 4.6 The effect of immobilisation stress and anti-IL-6-antibody treatment on GS activity in skeletal muscle of Wistar rats. GS activity was determined by measuring the formation of γ-glutamylhydroxamate in a reaction whereby glutamine is formed from glutamate, ADP and hydroxylamine. Results are expressed as means ± SEM, n = 39. Statistical analysis: Two-way ANOVA with Fischer's LSD as post test. *(#P<0.001).* Abbreviations: CP, Control Placebo; CA, Control Antibody; IP, Immobilisation Placebo; IA, Immobilisation Antibody.

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The immobilisation stress caused a significant up-regulation of GS activity in both stressed groups (main effect of stress with post hoc results presented in Fig 4.5: CP vs IP, $p<0.0001$; CA vs IA, $p<0.0001$). These nearly-2-fold increases (CP vs IP: 1.81-fold; CA vs IA: 1.67-fold) in GS activity are in agreement with a report published by Vats *et al.* (Vats *et al.*, 1999), who indicated a 2-fold increase in GS activity in Wistar rats after exposure to 7 days of chronic intermittent hypoxic stress.

The antibody treatment caused no significant changes in GS activity in either the control or stressed groups. Table 4.2 summarizes the effects of the intervention on GS activity.

TABLE 4.2. *Percentage and fold increases in GS activity in skeletal muscle of Wistar rats after exposure to immobilisation stress and anti-IL-6-antibody treatment.*

	Stressed groups		Antibody groups	
	IP vs CP	IA vs CA	CA vs CP	IA vs IP
Muscle	+ 81%	+ 67%	+15%	+14%
Fold-induction	1.81	1.67	1.15	1.14

Footnote: % increase: calculation e.g. ((IP/CP)*100) - 100; fold-induction: calculation e.g. IP/CP

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4.4 Correlations

Pearson's correlation analyses were performed on the different sets of results to determine if any variables were associated with each other. This analysis was done with the goal of obtaining possible trends in the data not otherwise seen, but it must be cautioned that it is not common practice to correlate data of entire groups of rats in the ways presented below. In short, the correlation analysis was not expected to produce any meaningful biologically relevant information. However, it is considered necessary in terms of preparation for future studies.
Significant relationships were found for the following pairs of variables that seemed to be most significant with regards to the outcomes of our study:

A) Change in body mass (ΔBM) *vs.* all other parameters
B) GR (liver) *vs.* GR (muscle);
C) GR (liver) *vs.* TAT (liver);
D) GR (muscle) *vs.* TAT (muscle);
E) GR (muscle) *vs.* GS (muscle) and
F) TAT (muscle) *vs.* GS (muscle).

The change in body mass (ΔBM) is used as an overall parameter of organismal stress (relationship described in A) and is related to all other parameters measured in this study in the whole group (Table 4.3) and in two sub-groupings (Table 4.4). Our aim was to establish which parameters influenced ΔBM and to what extent the latter was influenced by these parameters.

As seen in Table 4.3, the higher corticosterone concentration was indeed associated with greater loss of body mass ($r = -0.43$, $p<0.01$), but by itself it is not the main influence. GR binding capacity (in the liver) has the most significant relationship with ΔBM ($r = 0.68$, $p<0.0001$), with GR binding capacity (in the muscle) showing less, albeit still significant influence on ΔBM ($r = 0.53$, $p<0.001$). Although the enzyme parameters (TAT and GS activities) showed significant

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influences on ΔBM (ΔBM *vs.* TAT_{liver}, r = -0.55, p<0.001; ΔBM *vs.* TAT_{muscle}, r = -0.46, p<0.01; ΔBM *vs.* GS_{muscle}, r = -0.58, p<0.001), it is at least one level of significance less than the correlation between GR_{liver} and ΔBM (r = 0.68, p<0.0001).

Table 4.3 *Correlations between the change in body mass and other measured parameters of Wistar rats after exposure to immobilisation stress and anti-IL-6-antibody treatment.*

Parameters	*r-value* Total Group (n=39)
Serum parameter	
ΔBM *vs.* [cort]	-0.43**
GR parameters	
ΔBM *vs.* GR_{liver}	0.68#
ΔBM *vs.* GR_{muscle}	0.53***
Enzyme parameters	
ΔBM *vs.* TAT_{liver}	-0.55***
ΔBM *vs.* TAT_{muscle}	-0.46**
ΔBM *vs.* GS_{muscle}	-0.58***

Significance: *P<0.05; **P<0.01; ***P<0.001; #P<0.0001
ΔBM, change in body mass; [cort], corticosterone concentration;
GR, glucocorticoid receptor binding capacity; TAT, tyrosine aminotransferase activity;
GS, glutamine synthetase activity; r, Pearson product correlation coefficient

With the GR being influenced by corticosterone and the enzymes being GR-inducible, the question arose as to why such differences in significance are found between serum, GR and enzyme parameters. One possible answer may relate to the effects of the antibody – we therefore wanted to determine whether the antibody treatment affected these relationships. For this reason we performed similar correlation analyses on the rats that did not receive the antibody (the 'Placebo Group') as well as rats who received the antibody (the 'Antibody Group'). The results are presented in Table 4.4.

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Indeed, Table 4.4 shows that the levels of significance in the relationships found in the placebo group either improves (ΔBM *vs.* GR_{liver}), declines (ΔBM *vs.* GS_{muscle}; ΔBM *vs.* TAT_{liver}) or falls away completely (ΔBM *vs.* [cort]; ΔBM *vs.* GR_{muscle}; ΔBM *vs.* TAT_{muscle}) in the antibody group. It should be taken into account that both these groups (Placebo *vs.* Antibody) received varying levels of stress, namely an injection (placebo or antibody) with or without immobilisation stress.

Table 4.4 *Correlations between the change in body mass and other measured parameters of the placebo-treated (Placebo Group) and antibody-treated (Antibody Group) Wistar rats after exposure to immobilisation stress and anti-IL-6-antibody treatment.*

Parameters	*r-value* Placebo Group (n=20)	*r-value* Antibody Group (n=19)
Serum parameter		
ΔBM *vs.* [cort]	-0.59**	-0.27^{NS}
Liver parameters		
ΔBM *vs.* GR_{liver}	0.65**	0.75***
ΔBM *vs.* TAT_{liver}	-0.68***	0.58**
Muscle parameters		
ΔBM *vs.* GR_{muscle}	0.51*	-0.43^{NS}
ΔBM *vs.* TAT_{muscle}	-0.74***	-0.18^{NS}
ΔBM *vs.* GS_{muscle}	-0.66**	-0.51*

Significance: NS, non-signifcant ; *P<0.05; **P<0.01; ***P<0.001.
ΔBM, change in body mass; [cort], corticosterone concentration;
GR, glucocorticoid receptor binding capacity; TAT, tyrosine aminotransferase activity;
GS, glutamine synthetase activity; r, Pearson's product correlation coefficient

Table 4.4 clearly demonstrates that an abolishment of IL-6 activity leads to a significant disruption of the stress response in terms of expected downstream relationship between change in body mass and glucocorticoids, which agrees with the hypothesis that IL-6 is necessary for the full-blown stress response. In only the placebo groups and not in the antibody groups, significant correlations were observed between ΔBM and GR levels for both liver and muscle, with the best relationship observed for liver GR. In fact, the relationship between muscle

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GR and ΔBM was no longer significant in the antibody group. A similar observation could be made for the relationships between ΔBM and TAT: these were significant in the placebo in both tissues, but in the antibody groups the relationship was no longer significant in muscle tissue. In contrast, the relationship between ΔBM and GS in the muscle was present in both groups, although to a lesser extent in the antibody group. It is not clear why the muscle would be more sensitive to the absence of IL-6, so that in this tissue expected relationships were not seen unless IL-6 was present. A further study is needed to support these preliminary findings and to uncover the mechanisms underlying the observations.

Fig 4.7 illustrates relationships B-F as described at the beginning of section 4.4. We set out to measure GR binding capacity in both skeletal muscle and liver tissue. Since this was the first time that the GR radioligand binding assay was employed to measure GR binding capacity of skeletal muscle tissue in our lab, it is of added significance that the receptor levels in these tissues show such a strong correlation (Fig 4.7A: $r = 0.73$, $p<0.0001$). Although GR has been measured in either the one or the other tissue before (Al-Mohaisen *et al.,* 2000; Sun *et al.,* 2002), no study to date has looked at GR expression in both these tissues after a psychological stress intervention and therefore our results are the first to report such a strong correlation in receptor binding capacity between these different tissues under the specific experimental conditions of immobilisation stress. We argue that, although skeletal muscle and liver have different metabolic functions, similar regulatory mechanisms may be involved in the regulation of GR expression after stress, hence such a strong relationship between GR binding capacities of these tissues. Also, the relevance of this positive relationship is justified at the level of other proteins that are responsive to GC-action in peripheral tissues.

As expected, the relationships between GR binding capacity and the activities of the GC-inducible enzymes are all strongly negative (Fig 4.7B-D). Based on

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previous reports, we expected TAT levels to increase during and after the stress intervention, via the increase of circulating corticosterone levels and down-regulated GR (Dubois *et al.,* 1995; Yi-Li *et al.,* 1989). Studies that support the decrease of GR after exposure to either stress or IL-6-Ab treatments, include the following: Sapolsky *et al.,* 1984; Reul *et al.,* 1990; Omrani *et al.,* 1980; Gomez *et al.,* 1996; Nishimura *et al.,* 2004; Masera *et al.,* 2000 and Dovio *et al.,* 2001. The increase in TAT activity, on the other hand, is supported by the following studies: Granner *et al.,* 1968; Yi-Li *et al.,* 1989 and Ramakrishnan *et al.,* 2002. Although the in vivo TAT response (both at mRNA and enzyme activity levels) happens rapidly after GC treatment (5.5 hours), the effects of GR regulation last much longer, even after removal of the hormone (Dubois *et al.,* 1995). This explains why we could measure an increase in TAT activity on the fifth day of our experiment, which was 24 hours after the last bout of stress. In terms of the effects of IL-6 on TAT activity, we suggest that IL-6 may either independently and directly decrease TAT activity (the anti-IL-Ab increased TAT activity) or prevent excessive increases that occur due to stress by interacting negatively with the GC response at some level that would have downstream effects in both liver and skeletal muscle.

In support of the first possibility are previous reports that IL-6 decreases TAT activity in rat hepatoma FAO cells (Guzdek *et al.,* 1995) and that IL-6c (a recombinant IL-6 chimera) dose-dependently reduced TAT activity in fetal hepatocytes (Zvibel *et al.,* 2004). Not much work has been done on the interaction between TAT and cytokines *in vivo* and therefore our work contributes significantly in this regard.

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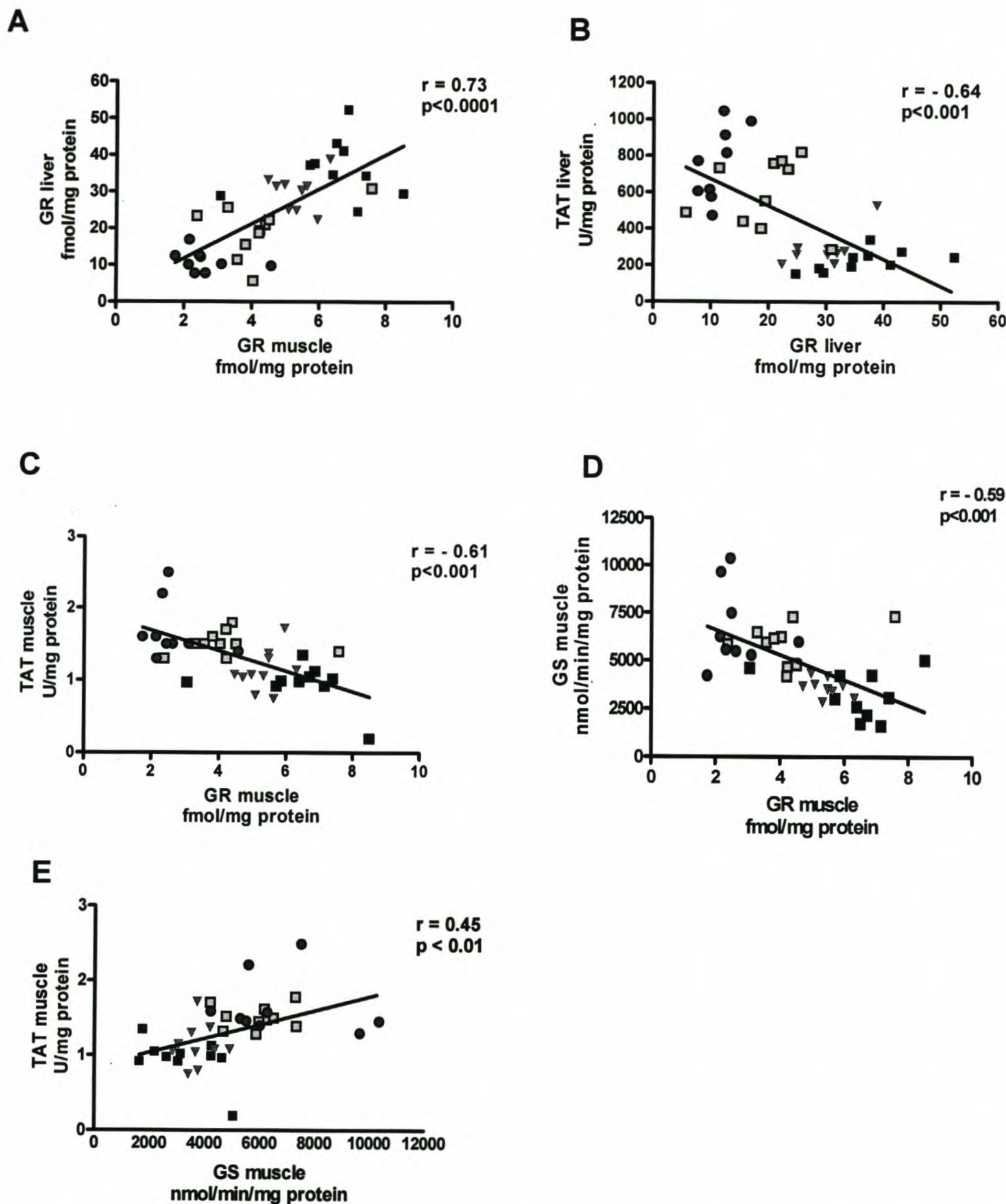

Figure 4.7 Correlation analyses between different parameters in skeletal muscle and liver of 39 Wistar rats after exposure to mild psychological stress (immobilisation) and anti-IL-6-antibody treatment. To aid in visualization of the distribution of the data points in each of the different experimental groups, the groups are shown in different colours: CP, Control Placebo (blue); CA, Control Antibody (red); IP, Immobilisation Placebo (yellow); IA, Immobilisation Antibody (green). The correlation coefficient (r) and p-values are shown alongside each graph. (A) GR_{liver} *vs.* GR_{muscle}; (B) GR_{liver} *vs.* TAT_{liver}; (C) GR_{muscle} *vs.* TAT_{muscle}; (D) GR_{muscle} *vs.* GS_{muscle}; (E) TAT_{muscle} *vs.* GS_{muscle}.

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In terms of TAT activity, the liver seems to be more sensitive to regulation by IL-6 than the muscle, since a significant antibody-dependent increase in TAT activity is measured in the liver (in the IA experimental group), but not in the muscle. TAT protein is much more abundant in the liver than in any other organ (Prieur *et al.,* 2001), which may explain the increased sensitivity of the liver to the anti-IL-6-Ab intervention. In support of a second possible mechanism of IL-6 action is the fact that the IA group did not have significantly elevated corticosterone (as assessed by the post-hoc test) and also lost the significant correlation between ΔBM and corticosterone that was evident in the placebo groups.

GS activity was measured in the gastrocnemius muscle while GR binding capacity was measured in the vastus. In order to perform optimal binding experiments, the two vastus muscles of each rat were pooled for one data point, hence the decision to measure GS activity in the gastrocnemius (the cytosol for the binding experiments could not be used for the assaying of GS activity). GS activity in gastrocnemius muscles of rats has previously been measured (Hundal *et al.,* 1990) while GR expression has previously been measured in both the vastus and gastrocnemius muscles (Czerwinski & Hickson, 1990). From this we thought it viable to correlate the GS activity with the expression of GR, which is shown in Fig 4.7D. Our results, as measured in gastrocnemius muscle, are mostly according to expectations. Stress and GCs are known to increase levels of GS activity (Vats *et al.,* 1999; Korn *et al.,* 1998), whereas increases in GS activity has been associated with decreased GR levels (Korn *et al.,* 1998). In murine astrocyte cultures, however, it has been shown that LPS inhibits GS activity via release of IL-6, IL-1 and TNF-α, also indicating that IL-6 partially inhibits GS activity (Hu *et al.,* 1994). Our results show that the anti-IL-6-Ab had no significant effect on GS activity and that increased GS activity was due to the immobilisation stress. The lack of an effect from the antibody can be explained by the facts that IL-6 has been shown to work in concert with other pro-inflammatory cytokines (such as IL-1 and TNF-α) and that high *in vivo* concentrations of a particular cytokine are often necessary for a significant effect.

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However, not many *in vivo* investigations have focused on the interaction between GS and pro-inflammatory cytokines, which makes it difficult to interpret our results in a more meaningful manner.

A significant relationship between the activities of TAT and GS is shown in skeletal muscle (Fig 4.7E). Although the activities of these two enzymes were measured separately in the vastus and gastrocnemius muscles, respectively, a positive relationship is interpreted in terms of the total protein catabolic activity during stress. This is the first study to indicate such a relationship, even though both enzymes have been studied extensively. In addition, a change in TAT activity in muscle has not been shown before since most investigations into TAT activity have focused on the liver as the main site of TAT activity. Our interest in this relationship is thus justified since we wanted to investigate the peripheral responses that happen in tissues after psychological stress. When the organism is faced with stress, glucocorticoid-induction of gluconeogenesis is known to occur, which is important in terms of survival. At the level of tissue responsiveness, this is related in terms of the amount of amino acids that are needed to serve as substrates for energy production. Since GS and TAT are both involved in amino acid catabolism, it becomes evident that such a positive correlation in their activities is justified in terms of the general stress response.

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CHAPTER 5 SYNTHESIS, CONCLUSIONS AND FUTURE STUDIES

The role of glucocorticoids in the stress response has been investigated for more than 50 years and since the identification of cytokines (about 30 years ago), the interaction between these two systems has become a separate field of scientific endeavour. To name a consequence of this, the field of psychoneuroimmunology (PNI) is a faster growing field of research than most others that study the link between physiology and behaviour. IL-1 is the first interleukin that is elevated in response to stress, but IL-6 has been identified as a mediator of IL-1 action (Ruzek *et al.,* 1997) and thus much attention has been placed on IL-6 as a highly pleiotropic cytokine. The basis of our study stems from our interest in the interaction between peripheral IL-6 and metabolic tissue-responsiveness to GCs after exposure to mild psychological stress. To our knowledge, we are the first to investigate *in vivo* tissue reponsiveness (GR expression and GR-inducible enzyme activities in both liver and skeletal muscle) in response to treatment with an IL-6-Ab.

Due to the 2 X 2 factor design of our experiment, we could separate the stress-induced effects from the antibody-induced effects, in addition to separating any interactive effects that might have occurred between them. To aid in explanation of both factors (stress *vs.* antibody), both factors will be discussed separately for each of the measured parameters as set out in Chapter 4.

Firstly, we set out to invoke a stress response in Wistar rats. Since studies on chronic stress have traditionally utilised quite extreme protocols such as restraint stress, we decided to investigate whether a mild psychological stress protocol could result in a similar stress response. This regimen of mild psychological stress resembles a more realistic form of stress experienced by humans on a weekly basis. Since our protocol was designed to be relatively mild, we chose to use a shorter duration. Therefore, the duration of our protocol was 4 consecutive

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days. We chose the morning hours (9 AM to 11 AM) to perform our experiments, based on information provided by Hu *et al.* which indicates that the plasma corticosterone levels are lowest during this time (Hu *et al.,* 2000). The same authors also indicate that the peak level of plasma corticosterone was reached within 60 minutes of immobilisation stress and lasted as long as the experimental duration (Hu *et al.,* 2000). For this reason we also chose 2 hours/day as the duration of the immobilisation stress sessions, which is also the duration chosen by Hu *et al.* (Hu *et al.,* 2000).

It has been shown that immobilisation stress causes significant reduction in body mass of rats (Hu *et al.,* 2000). The decrease in muscle mass (and consequently body mass) is related to the increases in corticosterone levels during stress (Herman & Cullinan, 1997). Taken together, the body mass and plasma corticosterone levels would indicate whether or not we achieved our first goal, namely that of eliciting a full-blown stress response. Indeed, our results show a decrease in body mass in both stressed groups (IA and IP) as well as a significant increase in the plasma corticosterone levels in group IP.

No significant increase in plasma corticosterone in group IA was measured compared to both control groups (CP and CA) and group IP. Elevations in IL-6 levels, in response to physical and psychological stressors, have been shown to lead to increases in GC concentrations, due to increases in CRH and ACTH levels (Zhou *et al.,* 1993; Kronfol *et al.,* 1992), which explains why the antibody treatment blocked a full-blown stress response in group IA. Thus, IL-6 is a necessary component in GC up-regulation during mild psychological stress *in vivo*.

With regard to the relationship between elevated corticosterone levels and loss in body mass, another significant observation is the positive relationship between these two parameters (change in body mass (ΔBM) *vs.* corticosterone levels – see Chapter 4) in the entire group of rats and the placebo group. This

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observation supports the existing notion that the change in body mass is related to the amount of psychological stress perceived by the individual, even if the stressful experience is as mild as 4 consecutive days with only 2 hr/d of immobilisation stress. This is consistent with previous reports that both increased plasma corticosterone levels and decreased body mass are found in rats that were exposed various kinds of stressors, for varying amounts of time (Harris *et al.,* 1998; Rybkin *et al.,* 1997; Hu *et al.,* 2000; Gursoy *et al.,* 2001). The decreases in body mass, in most cases, were detectable after only 3 days of e.g. restraint stress (Harris *et al.,* 2002). Future experiments may thus rely on body mass changes as a marker of stress perceived by experimental animals. It is noteworthy to mention that we do not observe the relationship described above in the group of rats treated with antibody. We think that this may be due to the larger number of samples that yielded non-detectable levels of corticosterone in groups CA and IA, which on the one hand influences the correlation, but on the other hand indicates the important role of IL-6 in the corticosterone response to stress.

The immobilisation stress caused a most pronounced down-regulation in GR binding capacity in both liver and skeletal muscle of the rats. This is a significant finding, since most previous studies concentrated on either one or the other of these tissues, but rarely both. When the GR binding capacities in both tissues were correlated, we found strong, positive correlation coefficients. A similar glucocorticoid regulatory mechanism may be active in both the muscle and liver during mild psychological stress, which is strongly reflected in the nearly identical pattern of GR binding capacity across both tissues in all the experimental groups. Therefore, our results (in peripheral tissues) support the negative feedback paradigm whereby increased corticosterone levels (due to stress) lead to decreased binding capacity of the GR.

As explained before, some previous studies support the idea that chronic stress leads to decreased levels and/or binding capacity of GR (Sapolsky *et al.,* 1984;

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Alexandrova & Farkas, 1992; Nishimura *et al.,* 2004). Others believe that GR levels and or binding capacity are either increased during chronic stress (Al-Mohaisen *et al.,* 2000; Sun *et al.,* 2002) or remain the same (Svec *et al.,* 1989; Lowy, 1991). We attempted to duplicate the methods of Al-Mohaisen *et al.* (Al-Mohaisen *et al.,* 2000), without achieving any success. We thus remain skeptical as to whether significant increases in GR binding capacity are measured after exposure to chronic stress. More recent reports also support the down-regulation of GRs, e.g. (1) decreased expression in neuronal GR in rats occurred following intrathecal IL-6 antiserum administration after chronic stress (Wang *et al.,* 2004) and (2) pro-inflammatory cytokines (TNF-α and IL-1β) in pathologically scalded rats was shown to be involved in the down-regulation of GRs (Liu *et al.,* 2002). These reports add further evidence that the down-regulation of GR expression serves to moderate the effects of the stress response, which in itself could be detrimental to the organism.

In addressing this discrepancy, another consideration will be evaluation of the role of the GR in subsequent tissue responses. Due to energy demands of the organism, the expression of TAT and GS enzymes need to be increased. Thus, the role of GRs would be the activation of the GREs (and/or GRUs) in these genes, which would ultimately result in increased breakdown of proteins (e.g. muscle) for the subsequent release of alanine and glutamine. The production and release of GCs from the adrenal lead to increased plasma levels of hormone, which ultimately feeds back on the hypothalamus to release less CRH and subsequently less ACTH. Thus, depending on the exact time during the stress response at which the plasma and tissue samples were collected, one might expect large differences in measured levels of both the GCs and GRs. This, in part, may explain some of the discrepancies with regard to the different reports regarding GR expression and/or binding capacity during stress states. However, the activated GR-complex is involved in regulation of many other proteins and enzymes, which adds to the list of complexities of the GC-GR system. Our results clearly indicate decreased GR binding capacity. Measurement of GR

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expression levels were beyond the scope of this project and should be considered in future interventions since it might shed additional valuable insight into our understanding of this complex matter.

The enzyme activities (TAT and GS) increased in liver and skeletal muscle even though the GR binding capacity decreased in both these tissues. This might appear perplexing since GR is known to activate the GREs in the promoter regions of the genes of these enzymes, and less GR should then lead to less enzymatic activity. One explanation could lie in the fractional occupancy of GR. The fractional occupancy is defined as the fraction of all receptors that are bound to ligand (Motulsky, 1996). Algebraically, it is shown as follows:

$$\text{Fractional Occupancy} = \frac{[ligand]}{[ligand] + K_d}$$

During stress, when the ligand (GCs) concentration is very high (many times K_d, the dissociation constant - an indicator of affinity between ligand and receptor, so that the value of K_d in negligible), it's fractional occupancy of the receptor equals 1.00. This means that all available receptors are occupied, which may ultimately have more GREs activated. During basal conditions, the K_d may be increased (resulting in the GC ligand having a lower binding affinity to the GR), which leads to a lower fractional occupancy. In short, increased enzymatic activities are feasible even with decreased GR binding capacity because the fractional occupancy of the GR by GCs is high during stress.

In addition to the effects of immobilisation stress, the IL-6-Ab treatment also led to decreased binding capacity of GR in both the livers and skeletal muscles of the animals. This is the first result of its kind in either liver or muscle tissue. However, IL-6 has previously been shown to increase GR levels in cells of Kaposi's sarcoma (Guo *et al.*, 1996), lymphoid (Rakasz *et al.*, 1993) and osteoblast-like cells (Dovio *et al.*, 2001). In an additional study published by a

group of neuroscientists, it was established that, in chronic neuropathic pain, a causal relationship exists between elevated levels of spinal IL-6 and neuronal GR expression (Wang *et al.,* 2004). Although it focuses on pain, the findings of this study (together with those of the studies mentioned above) correlate well with the data we present which indicate that IL-6 may up-regulate peripheral GR expression during psychological stress. In addition, several lines of evidence indicate that IL-6 and PKC play a prominent role in the expression of these neuronal GRs. It remains to be shown whether PKC (with or without IL-6) may mediate the same GR response in highly metabolically active peripheral tissues after psychological stress. Our study is the first to indicate that the IL-6-Ab leads to decreased levels of GR *in vivo* after psychological stress, since all the examples mentioned before are based on *in vitro* work. Furthermore, the reduction in GR binding capacity by the IL-6-Ab occurred with or without stress (Figs 4.4A and 4.4B: CP *vs.* CA; IP *vs.* IA). These data suggest a normal function of IL-6 is to maintain a basal level of GR binding capacity and in the face of stress, to partially counteract the GC effect (Masera *et al.,* 2000).

The significant relationship measured between ΔBM and GR binding capacity (in both liver and skeletal muscle) is shown in Table 4.3. Since loss of body mass is associated with increased levels of circulating corticosterone, GR binding capacity is also associated with levels of GCs, which is concurrent with common opinion regarding this matter. A new finding is that the ΔBM correlated best with GR binding capacity in the antibody group i.e. when a possible modulating effect of IL-6 was absent or at least diminished.

In many cases, tissue responsiveness to stress under specific experimental conditions (e.g. psychological stress or inflammation) has focused on receptor expression and subsequent enzyme activity. We specifically chose to study two enzymes that were known to be under GC control, viz. TAT and GS. We were particularly interested in whether neutralisation of IL-6 action could affect their respective activity levels. The immobilisation stress caused increased enzymatic

activities for both TAT and GS, in liver and muscle. However, it was only in the liver that TAT activity was increased significantly more in response to stress in the absence of IL-6. The latter implies that in the liver the presence of IL-6 can on the one hand antagonize GC-mediated enzyme upregulation, and on the other hand that there is a synergistic effect of IL-6 in downregulating the stress response similar to the effect of stress on GR binding capacity. In support of these results, a recent study has shown that IL-6c (a recombinant IL-6 molecule) reduces TAT activity in fetal rat hepatocytes (Zvibel *et al.,* 2004). To our knowledge, the latter is the only *in vitro* study to investigate TAT activity as a marker of tissue responsiveness to treatment with IL-6 or its recombinant analogues; and ours is the only *in vivo* study to indirectly assess the same thing through blocking of IL-6 action. In summary (with regards to enzyme activity) the effects of stress on protein catabolic activity were significant in liver and muscle, whereas a significant effect of the antibody could only be measured in liver. It remains to be seen if our study simply lacked the statistical power to show the same result in muscle, or if the two tissues are indeed differently responsive in this regard. In addition, the up-regulation of the protein catabolic enzymes is not inconsistent with the down-regulation of GR binding capacity, if it is assumed that total occupancy of GRs by GCs was higher under conditions of psychological stress.

In terms of the correlation analysis performed, we calculated significant negative relationships between ΔBM and TAT activity in both the total group of rats and the placebo group, but not in the antibody-treated group. This is interpreted to mean that loss of body mass is inversely proportional to the activity of TAT in liver and muscle. The lack of this relationship in the antibody-treated group is perplexing, and might be related to differential activation of the TAT gene GRE in the presence of the antibody. In the muscles, a relationship was observed between the ΔBM and GS, in the total group as well as the placebo- and antibody-treated groups. The fact that the relationship holds between ΔBM and GS, but not ΔBM and TAT, adds to this perplexing issue and further research is

needed to clarify this finding especially since we did report a positive relationship between the two enzymes' activities (Fig 4.7). Since these two enzymes have quite different roles in the cascade of protein catabolism, they have not both been investigated before in the same *in vivo* setting, but our interest was sparked because metabolic regulation of protein metabolism may determine whether or not an organism is capable of adapting successfully to chronic psychological stressors.

As mentioned throughout the thesis, much work has been done in terms of investigating HPA axis stimulation under various conditions, including acute stimulation by IL-6 (Raber, *et al.,* 1997). However, less is known about the chronic effects of IL-6 on the function of the HPA axis. Therefore, the aim of the next few paragraphs is to place the physiological significance of our study in context with previous work in the field, in an attempt to add evidence to the mechanism of peripheral IL-6-induced stimulation of the HPA axis under basal as well as stressed conditions.

In the rat, the sites in the the HPA axis where IL-6 could play a role in the stress response are the hypothalamus (Loxley *et al.,* 1991, 1993; Lyson *et al.,* 1991; Navarra *et al.,* 1991; Lyson & McCann, 1992; Spinedi *et al.,* 1992; Raber *et al.,* 1994; Yasin *et al.,* 1994; Kageyama *et al.,* 1995), the pituitary gland (Bateman *et al.,* 1989; Stephanou *et al.,* 1992; Muramami *et al.,* 1993; Sarlis *et al.,* 1993) and the adrenal gland (Muramami *et al.,* 1993; Gonzalez-Hernandez *et al.,* 1994). Rat IL-6 mRNA is found in the anterior pituitary (Vankelecom et al., 1989; Spangelo *et al.,* 1990, 1994) and is increased with chronic stimulation of the HPA axis by adjuvant injections (Stephanou *et al.,* 1992). Furthermore, IL-6 mRNA at the level of the anterior pituitary is under control of glucocorticoid feedback (Sarlis *et al.,* 1993). IL-6 itself stimulates ACTH secretion under *in vivo* and *in vitro* conditions (Bateman *et al.,* 1989; Spangelo *et al.,* 1994) whereas LPS activates the HPA axis in rats with PVN lesions implying that CRF-independent mechanisms are involved in the overall inflammatory response to LPS administration (Elenkov *et*

al., 1992). LPS also increases serum IL-6 levels and IL-6 mRNA in peripheral organs (Schöbitz et al., 1993), suggesting that IL-6 may be the mediating cytokine in the effects of LPS seen in the absence of the PVN . In summary, these reports support a direct effect of IL-6 on the pituitary or extrapituitary non-PVN pathways of HPA axis activation (Elenkov et al., 1992). In general, IL-6 seems to have a stimulating effect on the activity of the HPA axis.

In contrast, GCs are commonly known to have a suppressive effect on the HPA axis (Cole *et al.,* 2000). It was shown that the synthetic GC, dexamethasone, acts primarily on the GR in the anterior pituitary (Cole *et al.,* 2000), also that the inhibitory effects of dexamethasone did not take place at the level of the adrenal gland since the corticosterone response to an ACTH challenge was unaffected by dexamethasone administration (Cole *et al.,* 2000). Therefore, it is fair to conclude that circulating GCs generally attempt to inhibit HPA axis activity during stress by downregulating their own receptors.

In terms of the metabolic tissues we investigated, the anti-IL-6-Ab seemed to have similar effects, in different magnitudes, in both liver and skeletal muscle. This is interesting since many previous reports concentrated on either one or the other of these tissues and neglected to compare GR expression and/or enzyme activities between these important metabolic 'engines'. In addition, our study is the first *in vivo* study that investigates the peripheral metabolic effects of antil-IL-6-Ab on GC action. IL-6 has been shown to increase GR levels in osteoblast (Maseru *et al.,* 2000; Dovio *et al.,* 2001b), Kaposi's sarcoma (Guo *et al.,* 1996) and lymphoid cells (Rakasz *et al.,* 1993). One *in vivo* study in rats focused on central interaction between IL-6 and GR and found that IL-6 has a direct stimulatory effect on neuronal GR levels (Wang *et al.,* 2004). We report that the anti-IL-6-Ab decreases GR levels in the liver and skeletal muscle during mild psychological stress, indicating that IL-6 promotes GR levels, in agreement with the reports mentioned above.

Again, it should be mentioned that measurements of IL-6 concentrations were not obtained during the data collection process. This proves to be a handicap in interpretation since there is no indication of whether the antibody succeeded in neutralizing all IL-6 molecules. Results do indicate that the antibody succeeded in preventing a full-blown corticosterone response in the rats, but this does not prove in any way that IL-6 molecules have been bound by the antibody in circulation. It is suggested that this approach be taken in a follow-up study, since this would provide many insights into the role that IL-6 plays in modulation of GC action in the periphery.

In terms of speculation with regards to the duration of adaptation observed in the rats, the corticosterone levels are expected to normalize after a day or two after the last bout of stress. Body weight levels correlate well with changes in circulating GCs, thereby being expected to normalize within 3 days, permitted that no other major form of extrinsic or intrinsic stress is applied. Enzymatic activity levels are also expected to normalize within hours after removal of the stressor, although regulation by GR activation may prolong this normalization.

Taken together, our results indicate an antagonistic relationship between IL-6 and glucocorticoids and confirm the presence of cross-talk. In colloquial terms, IL-6 seems to act as the chemical inducer of a molecular brake on GC action during stress. This is especially evident in the tissue responses of our rats: the IL-6-Ab further decreased GR levels and further increased enzymatic activity (thus IL-6 itself would increase GR levels and decrease enzyme expression), whereas GCs are known to decrease GR and increase both TAT and GS activities (as explained in Chapter 2). Therefore, IL-6 may indeed be the key cytokine agent that modulates the GC-mediated stress response in metabolic tissues, possibly in order to partially limit protein breakdown. Furthermore, we suggest that IL-6 may both directly and indirectly influence enzymatic activity levels. It has been mentioned before that IL-6 is thought to act as a hormone, which supports the idea that it may exert direct effects on the activity of TAT in

the liver. In terms of indirect effects, the cross-talk paradigm between GCs and cytokines provide a basis for the suggestion that IL-6 may indeed modulate GC action and due to this, indirectly affect the activity levels of GC-inducible enzymes. In terms of the possible mechanisms as desribed in Fig 2.5, it is likely that IL-6 may indeed bind to nGRE sites by ways of composite or tethering mechanisms. It is also possible that IL-6 may compete with the other complexes for binding to the nGRE (competitive nGRE, Fig 2.5) and thereby repress activation of those enzymatic genes.

In conclusion, we confirm that protein breakdown occurs during chronic, mild psychological stress (manifested by the decrease in body mass) and that this is likely mostly due to protein catabolism mediated by the glucocorticoid system. Our data provides the first *in vivo* evidence that an absence of IL-6 modulates GC action, thus confirming the presence of cross-talk between the cytokine and endocrine systems in metabolically active peripheral tissues. Specifically, we show further down-regulation of both basal and stress-induced GR binding capacity in metabolic target tissues in the absence of IL-6. In addition, we report the first line of evidence to show that the activities of TAT and GS in mammalian organisms are positively related in terms of their catabolic abilities, especially under conditions of psychological stress.

This study has paved the way for further investigations into the peripheral interactions between the cytokine and glucocorticoid systems. Firstly, a more complete pattern of GR protein expression and distribution in peripheral metabolic tissues needs to be determined after exposure to chronic psychological stress. Knockout-mice and antibodies raised against cytokines both remain valuable tools for determining the exact mechanisms of interaction between endocrine and cytokine systems. However, use of antibodies holds the advantage that acute interference of the normal physiology can be investigated. In addition, this kind of research should encompass both central and peripheral investigations, since it is known that many actions of cytokines are stimulated by

interleukin-1 release in the central system. Importantly, it should be realised that the stress response is mediated by psychological, immunological, neural and endocrine systems as well as other target tissues, which should all be considered when planning such experiments. An ideal follow-up study will determine tissue-responses to different kinds of chronic stress, with biochemical and physiological analyses of a combination of the levels of CRF, ACTH, corticosterone, IL-1, IL-6, and GR. In this way, the peripheral tissue-responses can directly be correlated with central responses, which should provide a more holistic viewpoint of the integrated stress response.

Finally, in the peripheral metabolically active tissue, it would be valuable to attempt to ascertain the mechanism of IL-6 interference of GC action using molecular techniques to determine whether or not IL-6 acts directly, or indirectly on GRE's or GRU's.

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Appendix A

In this section, examples of calculations are shown for the different conversions of the values (obtained from the spectrophotometer and radioactive counter) to appropriate values. In all cases, the protein was diluted to a concentration of 2 mg/ml for muscle and 10 mg/ml for liver.

1. Glucocorticoid Receptor

The radioactive counter provides values in cpm (counts per minute). The table below shows the stepwise conversion to the appropriate units (fmol/mg protein) of one fictitious data point.

CPM	DPM	Ci	mol	mol/mg protein	fmol/mg protein
4123.4	11781.1	5.31E-09	6.394E-14	1.930E-15	1.9

CPM, counts per minute; DPM, disintegrations per minute; Ci, curie (measure of radioactivity)

2. Tyrosine Aminotransferase

The spectrophotometer provides absorbance values at a wavelength of 330 nm. The table below shows the stepwise conversion to the appropriate units (U/mg protein) of one fictitious data point. Three (3) absorbance values are obtained (10 minutes, 20 minutes, 30 minutes), from which a slope is determined (Absorbance per minute). A molar extinction coefficient of 19 900 $M^{-1}cm^{-1}$ is used to convert from absorbance values to molar units. One unit of enzyme activity is described as the amount of enzyme needed to convert 1 μmole of substrate per minute, at 37°C.

Abs/min	Abs/min/μg protein	M/hr/μg protein	μmoles/hr/ μg protein	U/μg protein/hr	mU/mg protein/hr	U/mg protein/hr
0.025704	0.000390	0.00000000196	0.0000002	0.000000196	196.0	0.196

Abs, absorbance; hr, hour

3. Glutamine Synthetase

The spectrophotometer provides absorbance values at a wavelength of 540 nm. The table below shows the stepwise conversion to the appropriate units (nmol/min/g protein) of one fictitious data point. One endpoint value is obtained per sample. The molar extinction coefficient used in the assay is 1.08 $M^{-1}cm^{-1}$.

Abs	Abs/mg protein	M/mg protein	M/min/mg protein	Activity (nmol/min/mg protein)
0.177	0.678	0.628	0.025	5023.4

Abs, absorbance; min, minute

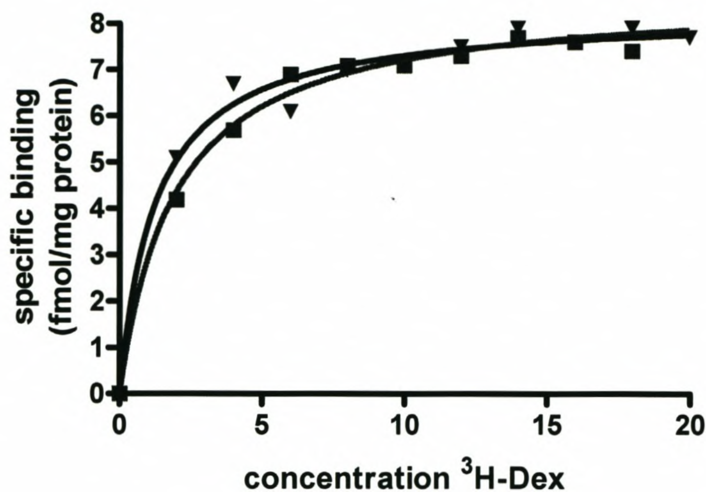
Appendix B

For purposes of method validation, saturation binding curves and scatchard plots were constructed. These analytical graphs are standard biochemical tools of assessment that form the basis for comparison to work previously published.

1) Saturation binding curves

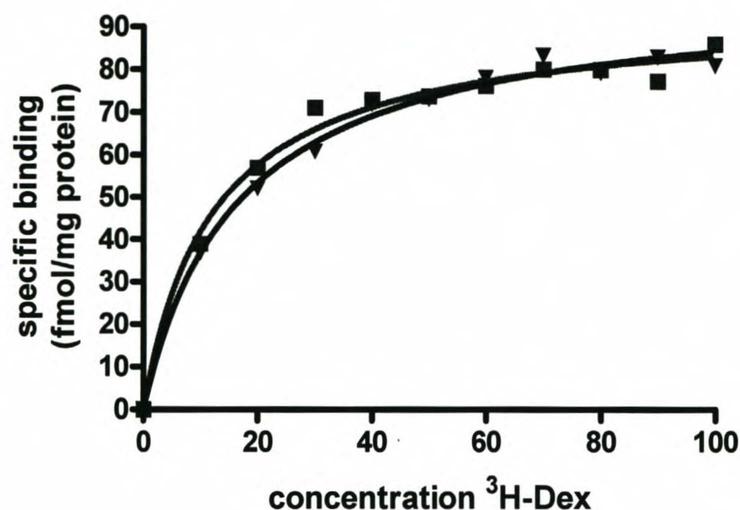
Saturation radioligand binding experiments measure specific radioligand binding at equilibrium at various concentrations of the radioligand. Analysis of this data allows one to determine receptor number and affinity. This kind of experiment is commonly analysed with Scatchard plots (see next section), and are therefore sometimes called "Scatchard experiments". The following graphs show data obtained from two single experimental attempts in both muscle and liver.

MUSCLE



Appendix Graph A Saturation binding curve in skeletal muscle tissue of a Wistar rat, showing data points from two separate experimental attempts (blue *vs.* red). Different concentrations of tritiated dexamethasone was used as radioligand and the measured binding capacity is expressed as fmol/mg protein. Specific binding is calculated by subtracting non-specific binding from total binding, as explained in Chapter 3 of this thesis.

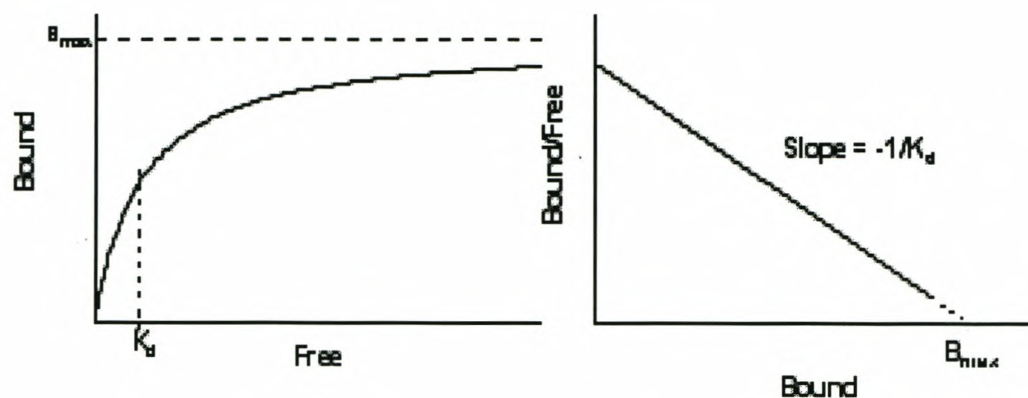
LIVER



Appendix Graph B **Saturation binding curve in hepatic tissue** of a Wistar rat, showing data points from two separate experimental attempts (blue *vs.* red). Different concentrations of tritiated dexamethasone was used as radioligand and the measured binding capacity is expressed as fmol/mg protein. Specific binding is calculated by subtracting non-specific binding from total binding, as explained in Chapter 3 of this thesis.

2) Scatchard Plots

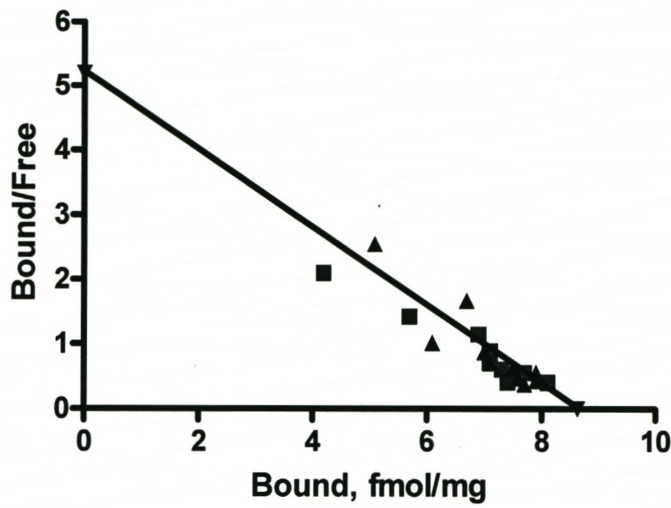
Previously, scientists transformed non-linear data into a linear form, and then analysed the data by linear regression. There are several ways to linearize binding data, including the methods of Lineweaver-Burke and Eadie-Hofstee. However, the most popular method to linearize binding data is to create a Scatchard plot, shown in the panel below.



In this plot, the X-axis is specific binding and the Y-axis is specific binding divided by free radioligand concentration. It is possible to estimate the B_{max} and K_d from a Scatchard plot (B_{max} is the X intercept; K_d is the negative reciprocal of the slope). However, the Scatchard transformation distorts the experimental error, and thus violates several assumptions of linear regression. The B_{max} and K_d values determined by linear regression of Scatchard transformed data may be far from their true values.

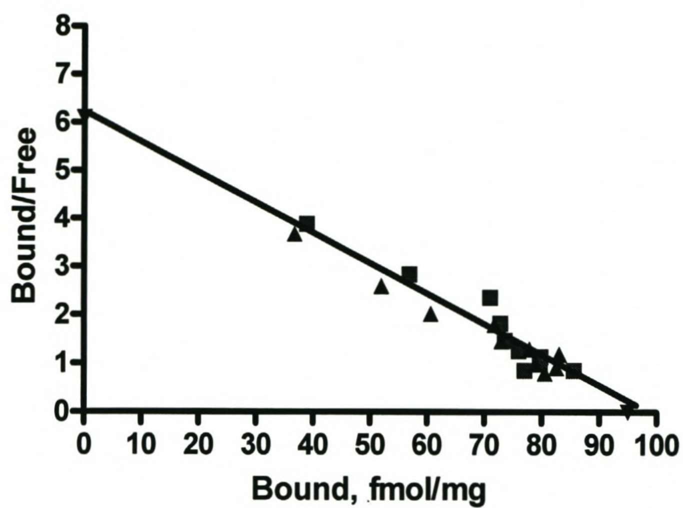
The derived Scatchard plots of Appendix Graphs A and B are shown below, for skeletal muscle and liver, respectively.

MUSCLE



Appendix Graph C **Scatchard plot of binding data** obtained from skeletal muscle tissue of a Wistar rat. The blue and red data points distinguished between data from two different experimental attempts. From this, the B_{max} and K_d values were determined to be 8.6 and 5.2 fmol/mg protein, respectively.

LIVER



Appendix Graph D **Scatchard plot of binding data** obtained from hepatic tissue of a Wistar rat. The blue and red data points distinguished between data from two different experimental attempts. From this, the B_{max} and K_d values were determined to be 95.0 and 6.1 fmol/mg protein, respectively.