An integrative approach to the effect of interleukin-6 on adaptation to restraint stress in rats

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DECLARATION

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Abstract

Bi-directional communication exists between HPA-axis activation and interleukin-6 (IL-6). However, the relative contribution of centrally *versus* peripherally secreted IL-6 remains unclear, especially under psychological stress conditions. We hypothesised that the HPA response to mild psychological stress is dependent on IL-6, both centrally and peripherally.

120 male Wistar rats were divided into four groups, depending on whether they received an anti-IL-6 antibody (Ab) (2µg/ml/kg body weight) or a placebo (sterile saline) injection and whether or not they were subjected to 1 hour of restraint stress for 1, 2 or 3 days. Rats were euthanized 24 hours after stress exposure.

Plasma corticosteroid (GC) levels remained significantly increased 24 hours after a single stress exposure (control placebo (CP) *versus* stress placebo (SP): $p < 0.05$). The undetectable plasma IL-6 levels evident across all groups may be explained by the short half-life of IL-6. Plasma IL-1β levels decreased when IL-6 was blocked in unstressed animals (CP *versus* CAb: $p < 0.05$), suggesting a role for IL-6 in the maintenance of IL-1β levels under tonic physiological conditions.

At tissue level, pituitary gland mass increased significantly at time point 2, independently of stress when blocking IL-6 (CAb: $p < 0.05$). This suggests that when normal homeostasis is threatened, immediate adaption or at least compensation may occur. It was observed that GR, IL-1β, IL-1βR, IL-6, IL-6R and $GABA_AR\alpha1$ showed no response to stress alone in the pituitary. It is therefore more likely that resistance to adaptation exists centrally. IL-1β and IL-1βR ($p < 0.05$) and $GABA_AR\alpha1$ ($p < 0.005$) expression increased in the CAb group in the pituitary, again suggesting a role for IL-6 under control conditions. In terms of the adrenal, blocking

IL-6 resulted in decreased glandular mass at time point 1, independent of stress (CAb and SAb: $p < 0.005$). The up-regulation in GR expression seen in CAb and SAb ($p < 0.05$) may be the effect of a compensatory mechanism to increase IL-6 dependent bioactivity of GCs. The fact that expression of IL-6, IL-6R, IL-1β and IL-1βR consistently increased in the Ab groups, and mostly in the *zona fasciculata* and *zona reticularis*, suggests that lack of local direct negative cytokine feedback occurred in response to very low plasma IL-6 levels and that this contributes more than GCs in the down-regulation of inflammatory cytokine release.

In conclusion, consistent effects of the Ab were apparent in the tissues investigated, even in control conditions, suggesting that IL-6 plays a role in the maintenance of basal homeostasis, including its regulation of the response to psychological stress. We found differential regulation in terms of cytokines and GCs when comparing peripheral *versus* central effects of stress and Ab, as well as the levels of cytokines in the blood compartment, compared to within tissues.

Opsomming

Daar bestaan twee-rigting kommunikasie tussen HPA-as aktivering en interleukin-6 (IL-6), allhoewel die relatiewe bydrae van sentraal *versus* perifeer afgeskeide IL-6 nog onduidelik is, veral gedurende sielkundige strestoestande. Ons hipotese is dat die HPA reaksie tot sielkundige stres afhanklik van IL-6 is, beide sentraal en in die periferie.

120 manlike Wistar rotte is in vier groepe verdeel, afhangende van of hulle ‘n anti-IL-6 teenliggaampie (Ab) (2µg/ml/kg liggaamsgewig) of ‘n plasebo (steriele soutoplossing) inspuiting gekry het, en of hulle onderworpe was aan 1 uur van vaskeer-stres vir 1, 2 of 3 dae. Rotte is 24 uur na blootstelling aan stres aan genadedood onderwerp.

Bloed kortikosteroïed (GC) vlakke het beduidend toegeneem binne 24 uur na ‘n eenmalige stres blootstelling (kontrole plasebo (CP) *versus* stres plasebo (SP): $p < 0.05$). Die onmeetbaar lae vlakke van IL-6 regoor al die groepe, kan verduidelik word na aanleiding van die kort half-leeftyd van IL-6. Bloed IL-1β vlakke het afgeneem in kontrole rotte wanneer IL-6 geblok is (CP *versus* CAb: $p < 0.05$). Dit kan beteken dat IL-6 noodsaaklik is vir die onderhoud van IL-1β vlakke gedurende basale toestande.

Op weefselvlak het die hipofise massa toegeneem by tydpunt 2 toe IL-6 geblok is, onafhanklik van stres (CAb: $p < 0.05$). Dit dui aan dat wanneer normale homeostase bedreig word, daar onmiddelike aanpassing of kompensasie plaasvind. Dit is opvallend dat GR, IL-1β, IL-1βR, IL-6, IL-6R en $GABA_AR\alpha1$ geen respons in terme van stres alleen in die hipofise getoon het nie. Na aanleiding daarvan is dit meer waarskynlik dat weerstand tot aanpassing sentraal bestaan. IL-1β and IL-1βR ($p <$

0.05) en $GABA_AR\alpha1$ ($p < 0.005$) uitdrukking in die hipofise het toegeneem in die CAb groep, wat weereens ‘n rol vir IL-6 onder kontrole toestande uitwys. In terme van die bynier, het die blok van IL-6 ‘n afname in massa veroorsaak by tydpunt 1, wat weer onafhanklik van stres was (CAb en SAb: $p < 0.005$). Die opregulering in die CAb en SAb groepe ($p < 0.05$), kan wees as gevolg van ‘n kompensasie meganisme om IL-6 afhanklike GC aktiwiteit te verhoog. Die feit dat die uitdrukking van IL-6, IL-6R, IL-1β and IL-1βR in die Ab groepe deurlopend verhoog was, en meeste in die *zona fasciculata* en *zona reticularis*, stel voor dat daar ‘n tekort aan plaaslike, direkte sitokien negatiewe terugvoering was, as gevolg van die merkwaardige lae bloed IL-6 vlakke en dat dit meer bydra as GCs in die afregulering van inflammatoriese sitokien vrystelling.

Ter opsomming, die konsekwente effekte van die Ab was beduidend in die betrokke weefsel, selfs onder kontrole toestande. Dit stel voor dat IL-6 ‘n rol speel in die onderhouding van basale homeostase, insluitende die regulering van die sielkundige stres respons. Ons het wisselende regulering in terme van sitokiene en GCs in die periferie *versus* sentraal gedurende stress en Ab toediening opgemerk, asook tussen sitokien vlakke in die bloed, in vergelyking met weefsel.

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Abbreviations

Ab	anti-IL-6 antibody
ACTH	adrenocorticotropin hormone
ANOVA	analysis of variance
AVP	adenosine vasopressin
BBB	blood brain barrier
BST	behavioural state system
CNS	central nervous system
CRH	corticotropin-releasing hormone
CS	cognitive system
CSF	cerebrospinal fluid
Fas	*zona* fasciculata
FITC	fluorescein streptavidin
GABA	gamma-aminobutyric acid
GC	glucocorticoid
Glom	*zona* glomerulosa
GR	glucocorticoid receptor
HPA-axis	hypothalamo-pituitary-adrenal axis
IL	interleukin
PBS	phosphate buffered saline
POMC	proopiomelanocortin
PVN	paraventricular nucleus
ME	median eminence
Med	medulla

MR	mineralocorticoid receptor
Ret	*zona* reticularis
SAM	sympathetic adreno-medullary axis
SD	standard deviation
SEM	standard error of the mean
SNS	sympathetic nervous system
TNF-α	tumour necrosis factor-α

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Introduction

Research has revealed that cumulative levels of stress have a profound effect on health and longevity, to the extent that specific diseases, such as cancer, diabetes and heart disease, as well as psychiatric ill health, can be initiated or amplified by stress (Buam and Posluszny, 1999b, Pitman *et al.*, 1990, Turnbull and Rivier, 1999). Over the years, several attempts have been made to identify key physiological markers and modulators of stress. However, the physiological output of stress depends on many factors such as the subjective experience of the stressor, the nature and duration of the stressor, the degree of controllability, and genetically based inter-individual differences (Petrides *et al.*, 1997).

Once we have achieved a reference framework with regard to specific major physiological role players in stress and their interactions have been delineated, steps can be taken to monitor for the balance of these interactions and contain stress-induced responses, in order to circumvent the deleterious effects of stress on health. An added benefit of this more specific approach is that the subjective, possibly skewed results obtained from questionnaires employed in the investigation of psychological stress can be compensated for, in an attempt to gain a more accurate view on stress dynamics.

Recently, it has been reported that indicators of stress perception such as “hassles and uplifts” (hassles in this regard specifically refer to the frequent strains and stresses of daily living) significantly and independently predict the circulating levels of pro-inflammatory markers such as interleukin-6 (IL-6) in a healthy population, independent of sociodemographic, biological and related psychological measures (Jain *et al.*, 2007). Furthermore, in the same study, chronic negative appraisals were

associated with increased circulating inflammatory mediator levels and persistent positive appraisals with decreased concentration.

With this thesis, we aimed to probe the mechanisms involved in the cross-talk between the neuroendocrine stress system and modulators of inflammation. In the first two chapters, we provide a review of the related literature. This is followed by a description of methods (Chapter 3) and results (Chapter 4). Our interpretation of the results and the conclusion drawn, as well as some directions for future research, are presented in the final chapter (Chapter 5).

Chapter 1: Background

Psychological stress can be defined as a negative emotional experience accompanied by predictable changes that are directed either toward altering the stressful event or to accommodate its effects (Buam and Posluszny, 1999a).

The evaluative process after input of a stressful stimulus involves processing of stimulus-specific information, coding of the stressor's intensity and intermittency, processing the degree of controllability, real or perceived, and comparing the current situation to previous experiences (e.g. as being novel or not). However, some stress stimuli can lead to a stress response without drawing on this evaluative process per se (classified as physical/systemic- versus psychological/neurogenic/processive-stimuli) (Herman *et al.*, 2003). For the purpose of clarification, we will refer to all stressors that do not require limbic processing, such as inflammation, ether administration, and hypoxia, as systemic stress and those that do (restraint, foot-shock, inescapable shock, immobilization, exposure to predators, and exposure to a novel environment for example) as psychological stress in this thesis. The classifications for the nature of stress will be discussed in more detail later (see section 2.1). Below is a diagram indicating the main brain and adrenal areas and the pathways involved during the acute, psychological stress response (initiated by the stress stimulus). For more detail on the diagram, refer to the following sections in the background chapter.

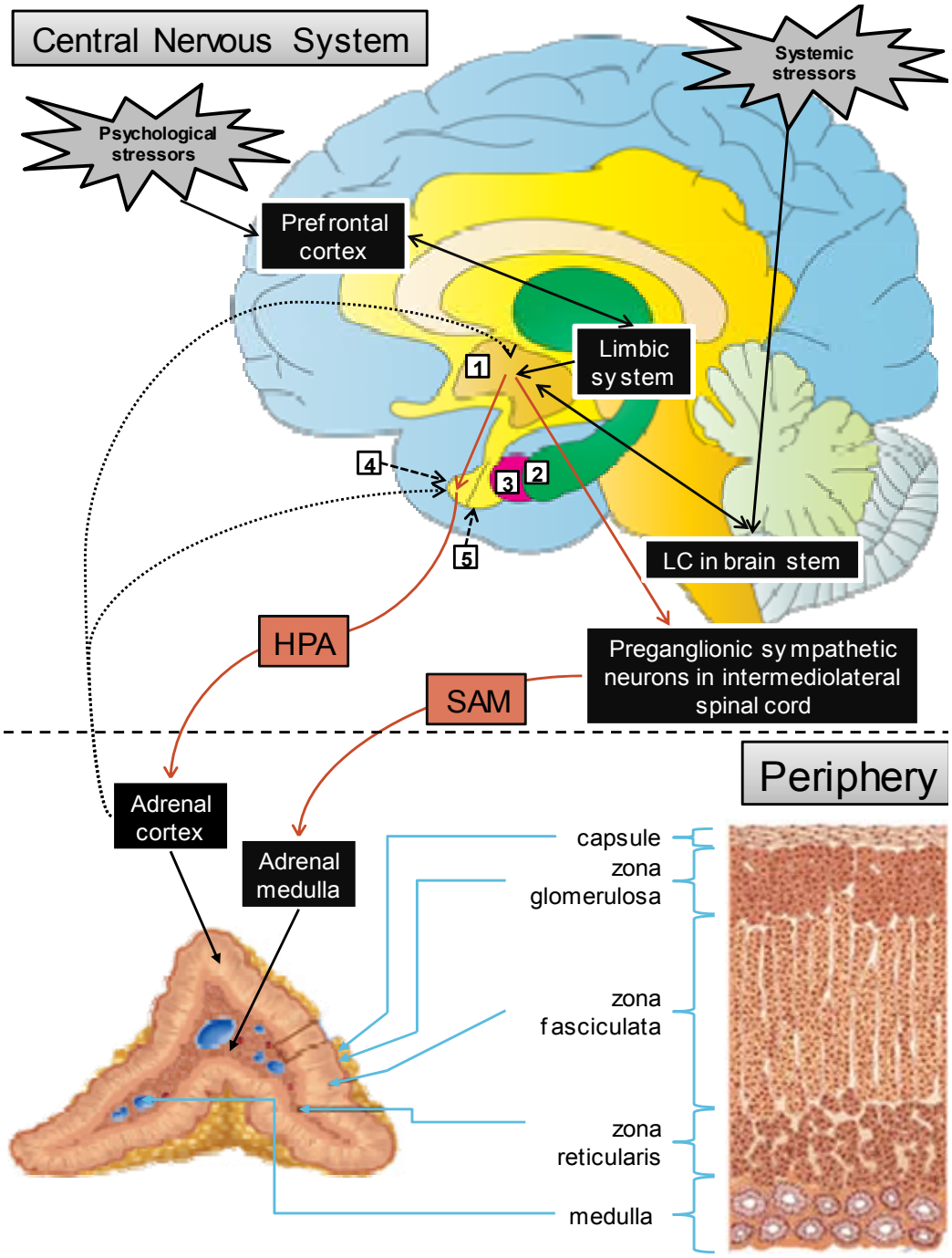


Figure 1: The main brain and adrenal areas involved in the stress response (negative feedback pathways indicated with dashed arrows). Stress stimuli are relayed to the CNS *via* the HPA-axis and the LC system regulating the SAM system. Numbers correspond to brain areas: 1) hypothalamus; 2) hippocampus; 3) amygdala; 4) anterior pituitary; 5) posterior pituitary. The diagram was adapted from the various sources. Abbreviations: LC, Locus coeruleus/norepinephrine system; HPA, hypothalamo-pituitary-adrenal axis; SAM, sympathetic adreno-medullary axis.

1.1 Relevant anatomical structures related to the stress paradigm

Stress integration involving the hypothalamo-pituitary-adrenal (HPA) axis employs 1) pathways converging at the medial parvocellular paraventricular nucleus (PVN) of the hypothalamus, 2) the pituitary gland, and 3) the adrenal glands.

Firstly, processing of anticipatory stressors takes place in limbic brain regions such as the amygdala, hippocampus, and prefrontal cortex which all innervate the PVN of the hypothalamus, the crucial locus for collection of stress stimuli (Lozovaya and Miller, 2003). The hypothalamus provides the interface between the perception of psychological stress and the regulation of downstream homeostatic processes (Lovallo and Thomas, 2000). The PVN also receives input from the periphery (as well as the cerebro-spinal fluid) via blood-borne factors such as glucocorticoids (GCs) crossing the blood-brain barrier (BBB). Blood-borne factors may also reach the PVN *via* the median eminence which is a BBB deficient region.

Two main physiological brain barriers exist: the vascular blood brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSF) which consists of a single layer of epithelial cells separating the choroid plexus blood from the cerebrospinal fluid (CSF) (Rapoport, 1976).

The vascular BBB is made up of a continuous monolayer of non-fenestrated endothelial cells connected by tight junctions (Rabpoport, 1976) which are both inside and outside the central nervous system (CNS), with a luminal surface facing the blood stream and an abluminal surface facing the brain interstitial fluid (Banks *et al.*, 2009). The luminal and abluminal membranes have different lipids, receptors, and transporters which cause the BBB to be polarized, enabling it to receive signals from one compartment and secrete mediators into the other. With the exception of

circulating immune cells that can cross the BBB, all other cell types are fixed in locations either inside or outside the CNS (Banks *et al.*, 2009).

Secondly, the HPA-axis is initiated via the parvocellular neurons of the PVN projecting towards the median eminence (ME) and releasing corticotropin releasing hormone (CRH) into the hypopheseal portal vessel (Lozovaya and Miller, 2003) which then reaches the pituitary gland. The pituitary gland consists of an anterior, glandular adenohypophysis with corticotrophs responsible for the secretion of adrenocorticotropin hormone (ACTH), and the posterior, neural neurohypophysis which is comprised of the axons of hypothalamic neurons (Childs, 1992).

Thirdly, ACTH targets the adrenal glands where it stimulates the release of GCs from the adrenal cortex. The adrenal glands are comprised of a large cortex region and a smaller (about 10% of the adrenal) fairly homogeneous inner region called the medulla. The cortex can further be subdivided into three concentric zones. From the surface inwards, the first zone is the thin *zona glomerulosa* and it is responsible for the synthesis of mineralocorticoids such as aldosterone. The middle zone is the thick *zona fasciculata* which produces GCs but also overlaps in hormone production with the inner thin *zona reticularis,* producing sex steroids as well as GCs (Young and Heath, 2004).

The adrenal medulla consists of mostly chromaffin cells that respond to surrounding adrenaline- (80%) and noradrenalin-producing cells, capillaries and venules. Chromaffin cells are derived from neural crest cells and are innervated by preganglionic sympathetic fibres (Young and Heath, 2004).

The basic view of cell types and regions within the adrenal gland of many mammals (including humans) as stated here, has subsequently been modified by data from a

number of researchers. For example, Bornstein *et al*, 1998 has found immune cells in the adrenal cortex and chromaffin cells in all the zones of the adult adrenal gland. Also, adrenocortical cells (especially in the rat *zona glomerulosa*) are able to synthesize additional molecules such as cytokines and contain TNF-α-, IL-1β-, and IL-6-mRNA (Bornstein and Chrousos, 1998, Lozovaya and Miller, 2003). The regulation of TNF-α remains unclear in the adrenal gland (Turnbull and Rivier, 1995). In the human adrenal gland, IL-6 expression is predominantly in the *zona glomerulosa* and IL-6 receptor (IL-6R) in the *zona reticularis* and *zona fasciculata in vitro* , although IL-6 protein and receptor are also co-expressed throughout the gland (Path *et al.*, 1997).

Therefore, the adrenal medulla and cortex are not separate entities as the textbook view holds, but rather exhibit bidirectional communication and receive input from the nervous and immune system. For example, intra-adrenal immune cells and cells of the medulla are a source of extrahypothalamic CRH and extrapituitary ACTH. This implies that GC production can proceed without the presence of pituitary ACTH (Bornstein and Chrousos, 1998).

Under conditions of chronic stress, one can discriminate from GCs released either as a result of central activation of the HPA-axis or directly from the adrenal cortex. Under these conditions, normal or below normal range ACTH levels do not correspond to the chronically elevated concentrations of GCs and the morphological changes in the adrenal gland, as there exist extrapituitary mechanisms of adrenal regulation (Bornstein and Chrousos, 1998) (section 2.2.2).

1.2 HPA-axis regulation

1.2.1 Cytokine interaction

Tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) stimulate the production of each other and that of IL-6 (Dinaerello, 1991) but IL-6 inhibits the production of both TNF-α and IL-1β (Nukina *et al.*, 1998a, O'Connor *et al.*, 2003, Schindler *et al.*, 1990). These pro-inflammatory cytokines can stimulate the HPA-axis independently or synergistically, either *via* extra-pituitary stimulation of ACTH most likely from lymphoid cells and the adrenal, or by acting directly on the appropriate brain regions (Eskay *et al.*, 1990). IL-6 activates the HPA-axis by enhancing the release of CRH or other substances that require the presence of CRH (Naitoh *et al.*, 1988). All three cytokines also have autocrine effects (Eskay *et al.*, 1990). TNF-α share many biological activities with IL-1β (responding to many of the same immune challenges) and both cytokines stimulate IL-6 and ACTH secretion, although their extra-pituitary site may differ (Eskay *et al.*, 1990, Sharp *et al.*, 1989).

Differential regulation of IL-1β, TNF-α and IL-6 by GC suppression can be demonstrated by using the lipopolysaccharide (LPS) model. When making use of this model in a study, LPS (a component of a bacterial cell wall) is administered in order to observe the effect this antigen exerts on the secretion of particular cytokines. One such study in mice (Zuckerman *et al.*, 1989) suggests that LPS challenge results in the acute activation of the HPA-axis *via* TNF-α, followed by its counterpart, IL-1β. However, in this study, IL-1β remained elevated for 24 hours post-LPS, whereas TNF-α had returned to control levels after 3 hours. This difference in disappearance rate was attributed to a biased corticosterone feedback system since the rate of corticosterone increase was similar to that of TNF-α, but

corticosterone remained elevated 24 hours post-LPS. However, differences in receptor dynamics were not assessed in this study, which could also have contributed to the differential degree of GC suppression observed.

1.2.2 Endocrine feedback

In addition to the differential sensitivities of cytokines to GC feedback, the HPA-axis may also be more or less sensitive to GC feedback, depending on the type and duration of the stressor. More specifically, GCs inhibit corticotroph function indirectly by inhibiting hypothalamic CRH and adenosine vasopressin (AVP) expression and release, as well as directly by inhibiting proopiomelanocortin (POMC) transcription for ACTH secretion by the pituitary corticotrophs (Aguilera, 1998). ACTH is a polypeptide tropic hormone which, under conditions of stress, is regulated by CRH, catecholamines, and AVP (Rivier and Vale, 1983).

Stress-related stimuli are relayed to the CNS, which is comprised of two systems: the CRH system (acting synergistically with AVP to regulate the peripheral activities of the HPA-axis) and the locus coerauleus-norepinephrine (LC-NE)/sympathetic neuron system of the hypothalamus and brain stem (regulating the systemic/adrenomedullary sympathetic nervous system (SNS). Activation of one system leads to activation of the other *via* CRF neurons synapsing onto α_1-noradrenergic receptors (Elenkov and Chrousos, 1999). The serotonin and cholinergic systems of the brain stimulate CRF, AVP and noradrenergic neurons while the gamma-aminobutyric acid-benzodiazepine (GABA-BDZ) opioid peptide systems, GCs, as well as ACTH and CRH themselves inhibit these effectors of stress (O'Connor *et al.*, 2000).

CRH, along with AVP, is primarily responsible for initiating the stress response by travelling down its site of production within neurons in the PVN of the hypothalamus, to the external layer of the median eminence. The PVN is comprised of two subdivisions: the magnocellular PVN (mPVN) which together with the supraoptic nucleus (SOP) produce AVP and release it from neurons in the posterior pituitary; and the parvocellulular PVN (pPVN) containing CRF neurons which release CRH into the hypophyseal portal circulation (Turnbull and Rivier, 1999). From here, it is released into portal blood where it gains access to one of its two plasma membrane receptors (CRH-R type 1) that reside on the corticotrophs in the pituitary (Turnbull and Rivier, 1999).

Both CRH and AVP control ACTH production and release from corticotrophs but through separate receptors and signalling pathways. Also, during chronic stress, CRH expression scales down while AVP expression increases, allowing ACTH to be released when an organism is exposed to a novel stressor (Miller and O'Callaghan, 2002). The differential roles played by AVP and CRH under different types of stress conditions may be attributed to a proportional release of these hormones, depending on their site of release from the PVN (Aguilera, 1998).

It is then reasonable to suggest that AVP is a likely candidate for maintaining corticotroph responsiveness under chronic stress conditions, in effect bypassing the inhibitory effects of GCs by its increased expression in parvicellular neurons, by potentiating the effects of CRH, and by increased binding in the pituitary (Makino *et al.*, 1995). In contrast, acute stress was reported to decrease pituitary CRH-R mRNA levels, only transiently after the initiation of stress, followed by an increase at 4 hours after initiation. In the case of immobilization, these increases in mRNA levels were also accompanied by increases in binding (Rabadan-Diehl *et al.*, 1996).

Opposite to their central effects, CRH and AVP have pro-inflammatory actions at local inflammatory sites, with CRH neurons and receptors exhibited in the adrenal gland, thymus, spleen, lymphocytes and other leukocytes. Even though CRH is not detected peripherally during stress, CRH levels at inflammatory sites may display concentrations similar to those found in the hypophyseal portal system (O'Connor *et al.*, 2000).

In conclusion, the regulation of CRH and AVP in the PVN varies, depending on the duration of stress exposure, as well as the regulation of CRH-R expression, which is controlled by the synergistic actions of CRH, AVP, and GCs. Therefore these mediators participate in the responsiveness of the HPA-axis.

1.3 Neuro-endocrine immune loop

The orthodox observation on the HPA-axis involves the following:

Firstly, a stressor is assessed by the prefrontal cortex, after which the amygdala is put on alert and activates the axis. The brain stem influences state of arousal whereas the integration of external stimuli and the appraisals thereof converges on the mpPVN, which secretes CRH into the hypopheseal portal vessel reaching the anterior pituitary. CRH binds to its receptors in the anterior pituitary which, in turn, secretes and releases ACTH into the circulation. ACTH then activates the synthesis and release of GCs from the adrenal cortex (Feldman *et al.*, 1995).

This view on the HPA-axis may be modified by incorporating the model of Swanson (2003). According to this model, there are three different systems that determine the output by the HPA-axis: Firstly, the **sensory system** (SS) relays stress related stimuli (internal or external) to the cerebral cortex where perception is created, after

which it travels to the limbic system. After awareness of an emotion has been created in this area, it feeds back to the cerebral cortex. Secondly, a **cognitive system** (CS) resides within the cerebral cortex which determines which voluntary responses will be engaged. Thirdly, descending pathways from the limbic system reach the **behavioural state system** (BST) which comprises the hypothalamus, limbic system and brain stem. The BST also allows for motor output to govern skeletal muscle movement and visceral responses such as the actions of smooth and cardiac muscles and endocrine and exocrine glands. Finally, these physiological or behavioural responses feed back to the SS which initiates the pathway once again by communicating with the CS and BST (Swanson, 2003) (see Fig 1).

Apart from the behavioural and visceral responses mentioned above, immune modulators such as cytokines also relay information to the SS, constituting the neuroendocrine-immune loop. Evidence in support of this loop stems from knowledge that the immune and neuroendocrine system cells share common ligands and hormone and cytokine receptors (Turnbull and Rivier, 1999), immune cell functions can be modulated by hormones and neuropeptides (Buckingham *et al.*, 1996), immune cells can secrete ACTH (Turnbull and Rivier, 1999), and the immune system is innervated by noradrenergic sympathetic nerve fibres (Chikanza and Grossman, 1996). For example, according to early studies, activation of the ventral noradrenergic tract (responsible for noradrenergic innervation of the hypothalamus, carrying the axons of noradrenergic neurons with cell bodies in the brain stem) by cytokines released from an immune or inflammatory lesion and stimulating local sensory afferent fibres, results in neural projections sent from the nucleus tractus solitarius to the PVN (Gaykema *et al.*, 1995, Laye *et al.*, 1995). In addition, this tract is also activated by plasma IL-1β *via* stimulation of perivascular cells in the adrenal

medulla (Buckingham *et al.*, 1996, John and Buckingham, 2003). We now turn our attention to the action of cytokines as part of the neuro-endocrine immune loop.

Cytokines are polypeptides or glycopeptides and are produced by various cells in both the periphery and CNS. Their receptors are located on membranes of a variety of cells, including immune cells, glandular cells, neurons, astrocytes, microglia, cerebrovascular endothelia, neuroblastoma cells, and glioblastoma cells (Fink, 2000, Turnbull and Rivier, 1999). Cytokines comprise the interleukins (ILs) and TNF. Designations for the ILs used to depend on which cell type was identified to secrete it (e.g. monokines secreted from monocytes, lymphokines secreted from lymphocytes and cytokines secreted from non-lymphoid cells) (Eskay *et al.*, 1990). Today the ILs are referred to as cytokines irrespective of their origin. Over the years, *in vitro* as well as *in vivo* studies have indicated many different roles for various cytokines at the level of the hypothalamus, pituitary and adrenal glands.

When referring to the above mentioned stress-related systems model, inflammatory cytokines such as IL-1β, IL-6 and TNF-α can have a direct effect on the CNS *via* acting on the hippocampus for instance (controlling behaviour) or indirectly through actions at different levels of the HPA-axis (Fink, 2000). Studies investigating the activation of the HPA-axis by cytokines administered either centrally of peripherally, found that activation is attributable to stimulation at or above the level of the hypothalamus (Turnbull and Rivier, 1999). Because cytokines are relatively large molecules (e.g. human IL-6 amounts to 21-28 kD and IL-1 to 15-25 kD), they require mechanisms to cross the BBB in order to exert their function. Some of these mechanisms do not necessarily involve crossing the BBB itself, but bypassing this obstruction *via* the following methods:

- Cytokines, IL-1β in particular , causes the release of prostaglandins (PGE_2, PG_{12}), catecholamines, serotonin, histamine, eicosanoids, and nitric oxide by binding to their receptors on endothelial cells outside the BBB, which then indirectly activates the HPA-axis *via* CRH secretion from the median eminence (Imura *et al.*, 1991, Kronfol and Remick, 2000, Sternberg *et al.*, 1992, Turnbull and Rivier, 1999).
- Cytokines can passively enter into leaky parts (e.g. sites where local inflammation is present) or parts devoid of the BBB (*via* fenestrated capillary endothelium without tight junctions) called circumventricular organs, such as the organum vasculosum of the lamina terminalis, PVN, area postrema, median eminence, posterior lobe of the pituitary, and the CeA of the amygdala (Anisman, 2008, Imura *et al.*, 1991, John and Buckingham, 2003, Kronfol and Remick, 2000, Lozovaya and Miller, 2003, O'Connor *et al.*, 2000, Turnbull and Rivier, 1999).
- Transcellular, saturable transport mechanisms (carrier-mediated transporters, receptor-mediated transcytosis, and efflux transporters) for IL-1α, IL-6, and TNF-α exist (Banks *et al.*, 1995, Kronfol and Remick, 2000, Miller and O'Callaghan, 2005).
- Signalling to the brain (and causing, for example, IL-1β production in the brain) may also be facilitated by cytokines binding to their receptors on peripheral paraganglia which synapse on afferents such as the abdominal vagus nerve (the 10^{th} of the paired cranial nerves reaching into the abdominal cavity where it innervates the viscera) (Anisman, 2008, Fleshner *et al.*, 1995, Haddad *et al.*, 2002, Kronfol and Remick, 2000, Miller and O'Callaghan, 2005).

- To relay cytokine-related messages within the brain *via* astrocytes, microglia and neurons in the brain synthesizing IL-1β, IL-6 and TNF-α (Anisman, 2008, John and Buckingham, 2003, Kronfol and Remick, 2000, Lozovaya and Miller, 2003, Miller and O'Callaghan, 2005, O'Brien *et al.*, 2004).
- Up-regulating of adhesion molecules such as ACAM-1 and VCAM-1 increases adhesion of circulating T lymphocytes to the endothelial lining of the BBB (Anisman, 2008, O'Brien *et al.*, 2004). Lymphocytes crossing the BBB can produce IL-1β, IL-6 and TNF-α (Lozovaya and Miller, 2003).
- Cells that make up the BBB can also secrete cytokines (Banks *et al.*, 2009).

Gathered from the knowledge contained in chapter 1, it is clear that the response to stress is complex in terms of specific interactions between mediators of stress under, specific conditions, and in specific areas of the body. Much is still unknown and therefore future studies should be directed towards filling the gaps in our understanding of stress responses. The next chapter deals with what is known from the literature pertaining to our chosen model of stress.

Chapter 2: Literature review

2.1 Classification of stressors

The literature is vague in terms of categorising different types of stressors as is evident in the following section.

Psychological stress exposure elicits many of the same responses than that occurring under conditions of infectious and inflammatory stimuli (adipsia, aphagia, fever, HPA-axis activation, reduced social interaction and changes of acute phase proteins). However, studies have shown that psychological stressors such as restraint, immobilization, or exposure to an open field elicit differential pathways than those activated in response to systemic stressors such as ether or intraperitoneal injection of LPS, with LPS activating the central subnuclei of the amygdala and restraint acting on the medial subnuclei (Day *et al.*, 1999, Emmert and Herman, 1999). Strenuous acute physical activity activates the sympathetic nervous system and is regarded as a model for inflammation-like processes (Shepard and Shek, 1998).

Another discrepancy with regard to the effect of different classes of stressors, resides in the notion that while some types of stressors such as inescapable shock, footschock, immobilization, restraint, and open field exposure, enhance IL-1β, and IL-6 action, others such as brief handling, decreases these cytokine levels (Briski and Gillen, 2001, Goshen and Yirmiya, 2009).

One group distinguished two types of stressors: those that they termed to be neurogenic in nature and of physical origin such as immobilization stress, inescapable shock, and formalin injection and those which are psychogenic

stressors of only psychological origin (Plata-Salama *et al.*, 2000). Other studies regard immobilization and restraint stress to be psychogenic in origin, requiring higher order processing (Herman *et al.*, 1998). In our opinion, whether restraint stress is classified as 'psychogenic', depends on the severity of the model.

The ultimate end product of any type of stress insult is activation of the HPA-axis, but the way in which the axis is being activated differs for systemic and psychogenic stressors with regard to the brain areas required for stimulus processing. Systemic stressors activate the HPA-axis directly *via* brain stem relays, whereas psychogenic stimuli processing requires pathways to the limbic system for comparison to past stimuli (Herman and Cullinan, 1997).

In addition, there are two main realms of HPA activation occupying distinct pathways, although multiple pathways may be involved, especially if both classes of responses are simultaneously implicated (Anisman, 2008). This hypothesis was first introduced in 1951 in order to explain the notion that some stressors such as epinephrine, cold, and histamine still elicited a corticosterone response, even when the pituitary has been removed, and while others such as immobilization and sound relied on an intact pituitary to bring about a response.

The first class termed systemic stress pathways entails a real homeostatic challenge that is recognised by changes in somatic (cardiovascular tone, respiratory stress, pain), visceral (pain) or circumventricular sensory pathways (blood-borne cytokine or chemokine factors). These reactions are brought about by reflex pathways with afferents directly to the PVN originating in the brainstem and which are not affected by lesions of the limbic system. In an experimental set-up involving animals, these stressors constitute a direct threat to survival and include ether stress or severe

hypoxia, or cases where the (systemic) immune system is compromised (Herman and Cullinan, 1997).

The second class termed processive stress involve limbic stress pathways (reactions affected by lesions of the prefrontal cortex, hippocampus or amygdala) and higher-order sensory processing of multiple sensory modalities. These responses are produced either as a result of conditioned stimuli (a memory, environment) or when innate species-specific tendencies (recognition of predators, heights, and open spaces) are present (Herman *et al.*, 2003). These conditions have been simulated in experimental animal models of restraint, fear conditioning or exposure to a novel environment (Herman and Cullinan, 1997). Of note, prior to additional synapses between limbic sites, different types of processive stressors may employ different pathways, as seen in restraint stress which shows differential patterns of central *c-fos* mRNA induction than swim stress (this may be explained in terms of the amount of movement allowed with these stress regimes) (Herman and Cullinan, 1997).

Taken together, psychological (processive) stress may also have systemic components. This suggests that the response to psychological stress depends on the specific set(s) of sensory pathways employed, from different areas in the brain and body.

Other factors that need to be taken into consideration when assessing mediators of stress are based on observations on responses such as acceleration of heart rate, adrenal catecholamine secretion, and activation of the HPA-axis that vary in magnitude and/or duration, based on the nature, length of exposure, and/or intensity of psychogenic stress (Briski and Gillen, 2001, Kronfol and Remick, 2000). In addition, cytokine expression profiles differ with respect to the region of analysis in

the brain in immobilization, restraint, forced swim, and predator exposure models of stress (Briski and Gillen, 2001, O'Connor *et al.*, 2003). Also, the degree of controllability of a stressor influences serotonin output from the prefrontal cortex. However, the component of controllability does not seem to modulate corticosteroid secretion. When a stressor is first encountered, it cannot be determined whether the stressor is controllable or not, or brief or prolonged. Only as the stressor continues, do differences in HPA-axis control emerge (Anisman, 2008). It is therefore important to consider the nature and duration of a stressor and the brain region employed when assessing the effects of stress or response to a particular stressor.

Of interest, human studies have revealed subjects reacting differently to stressful stimuli, be it psychological or high intensity exercise and that there are high responders, exhibiting exaggerated HPA-axis responds in both stress categories (Cacioppo *et al.*, 1995, Petrides *et al.*, 1997, Sgoutas-Emch *et al.*, 1994). Furthermore, high responders to psychological stress were shown to also be prone to high responders with exercise, indicating a non-specific tendency for greater stress reactivity (Singh *et al.*, 1999). However, these differential responds to stress are not apparent when dealing with animal models of stress. These 'absence of responder sensitivity' levels may pose an advantage to the use of animals instead of human subjects in studies investigating the response to stress.

Some broad conclusions can be drawn from the section above by drawing on knowledge pertaining to specific pathways activated under different conditions of stress:

Systemic (physical) stressors activate firstly the HPA-axis *via* activation of noradrenergic cell bodies in the brain stem by IL-1β, which relays the stimulus to the

hypothalamus, ultimately up-regulating CRH secretion in the hypothalamus leading to GC secretion from the adrenal glands. The brain region acting as a sensory organ differs to the region employed under conditions of psychological stress (in which case the stimulus originates in the limbic system). Secondly, systemic stressors act directly on the adrenal gland to release GCs by the action of cytokines in circulation.

In the case of psychological stress, the first reaction to stress involves the sympathetic-adrenomedullary (SAM) system or sympathetic nervous system (SNS) (these terms are used interchangeably) which is employed as an early fight-or-flight response to stress. The cerebral cortex is responsible for labelling of psychological stressors as harmful and this stimulus is relayed to the hypothalamus from where a signal is sent to the adrenal medulla to secrete catecholamines, ultimately leading to effects such as increased heart rate, sweating, constriction of peripheral blood vessels and activation of the immune system (Axelrod and Reisine, 1984, Taylor, 2003).

Secondly, the HPA-axis is activated as a more delayed response to stress with afferents from within the CNS originating in limbic sites (once the stimulus has been compared to past stimuli) and from the periphery *via* the blood supply to circumventricular organs. The latter way of stimulating the HPA-axis by means of blood-borne cytokines, may be considered to form part of a feedback mechanism, more so than initiation of the HPA-axis (Zhou *et al.*, 1993).

Thirdly, central catecholamines increase cytokine levels in the brain *via* increased CRH action. Lastly, the adrenal gland alone also plays a role in the stress response to psychological stress via catecholamines from the peripheral sympathetic nervous

system, activated by CRH and/or prostaglandins, which ultimately increases cytokine expression in the adrenal gland.

2.2 Response to stress

Many factors influence the ability or capacity of an individual to adapt to stress. Tolerance and cross-tolerance (habituation) of the HPA-axis occur after repeated non-immunogenic homotypic stress exposures (Fernandes *et al.*, 2002, Garcia *et al.*, 2000, John and Buckingham, 2003, Melia *et al.*, 1994), although the degree of habituation depends on the duration, frequency and number of applications, and the timing of the blood sample (Fernandes *et al.*, 2002). Stress intensity also plays a role: the less intense the stimulus, the more prominent the habituation and with very intense stressors, there may be no habituation at all (Pitman *et al.*, 1987).

It has been found that under chronic restraint stress conditions, the duration and magnitude of ACTH and corticosterone responses are significantly blunted when an additional acute stimulus of the same type is applied (compared to the responses in a naive rat) (Fink, 2000, Hauger *et al.*, 1990, Ma *et al.*, 1998).

Contrary to the above scenario of cross-tolerance, if an acute, novel stimulus is applied to a chronically restraint stressed rat, the duration and/or magnitudes of ACTH and corticosterone are promoted compared to that of the naive rat (Bhatnagar and Dallman, 1998, Fink, 2000). Also, cross-tolerance of the HPA-axis does not hold under conditions of repeated heterotypic stress and rats subjected to restraint stress before receiving an acute LPS injection, show exaggerated CRH mRNA expression in the PVN. (John and Buckingham, 2003). Therefore, repeated exposure to one stressor can lead to an exaggerated HPA response to an additional heterotypic stressor (cross-sensitization) (Hauger *et al.*, 1990, Ma *et al.*, 1998, Pitman *et al.*,

1990). However, cross-sensitization does not occur in all types of stress situations (Chung *et al.*, 2000, Martı´ *et al.*, 1999).

Consequently, when investigating stress responses, it is advised to either explore the effects of a single, isolated (acute) stressor, or to take the role of habituation and sensitisation (depending on the novelty of the stressor) into account when assessing repeated stress effects.

2.2.1 The role of glucocorticoids

GCs have been generally thought of as inhibitory modulators of immune activity, preventing the immune system from overshooting under conditions of inflammation for example (although more recent work has revealed a more extensive role for GCs, depending on type of immune activity and the particular cells involved). It is now evident that the immune system can also regulate corticosteroid function by way of immune cells secreting molecules that indirectly down-regulate their own activity *via* increasing GC secretion from the adrenal glands (Turnbull and Rivier, 1999).

GCs exert their effects on the inflammatory cytokine system in various ways, including through suppression of gene expression, transcription, translation, post-translational processing, protein secretion, and cell progenitor proliferation and differentiation (O'Connor *et al.*, 2000). GCs inhibit pro-inflammatory cytokine production as well as the production of arachidonic-acid-derived pro-inflammatory substances such as leukotrienes and prostaglandins (O'Connor *et al.*, 2000). TNF-α, IL-1β and IL-6 production is inhibited by GCs to varying degrees, with TNF-α suppressed most (at physiological levels), IL-1β suppressed less and IL-6 displaying almost no sensitivity to inhibition and is in effect resistant to GC action (DeRijk *et al.*,

1997, Fink, 2000). This phenomenon may be explained by the differential actions of the type 1 and type 2 GC receptors (GRs).

The mechanism of action for GC suppression entails the inhibition of pro-inflammatory transcription factors such as nuclear factor-κβ (NF-κβ). Without any GC signals, NF-κβ is bound to IκBα and IκBβ which prevent NF-κβ from entering the nucleus. Once NF-κβ is activated by stressors such as viral infections, oxidants, cytokines, and antigens, IκB is released and NF-κβ enters the nucleus where it binds to the promoter areas of genes transcribing for more cytokines, enzymes and adhesion molecules, for example. GCs intervene with this process by binding to activated NF-κβ and by increasing the transcription of IκB (O'Connor *et al.*, 2000).

In conclusion, bidirectional communication exists between GCs and cytokines, with GCs inhibiting IL-1β, TNF-α and IL-6 production (albeit in varying degrees), while these cytokines in turn promote GC release from the adrenal. It is therefore necessary to consider both GC and cytokine responses to stress, as well as consequent interactions amongst different cytokines and between GCs and cytokines.

2.2.2 Pro-inflammatory cytokines and stress

Of all psychological stressors, the majority of reports have shown that immobilization and shock paradigms are the most likely to influence central IL-1β responses (as reviewed in Deak et al., (2004)). IL-1β expression increases in the hypothalamus after rats have been exposed to restraint and immobilization stress (Imura *et al.*, 1991, Kronfol and Remick, 2000). The consequent effects of this raise in IL-1β in different parts of the HPA-axis are evident in the following paragraphs.

IL-1β is produced both by activated monocytes and non-immune cells. Studies have located the site of action and the conditions under which IL-1β stimulate the HPA-axis by way of administering recombinant IL-1β for an acute period. It has been speculated that the increase in CNS or hypothalamic activity is the result of activation of noradrenergic cell bodies in the brain stem by IL-1β which, in turn, upregulates CRH secretion and biosynthesis in the hypothalamus (Eskay *et al.*, 1990, Imura *et al.*, 1991).

IL-1β also contributes towards a rise in plasma ACTH, which is more pronounced after intracerebroventricular than intravenous injection of IL-1β, suggesting that the site of action for IL-1β is in the CNS (Imura *et al.*, 1991). These findings have led researchers to believe that the site of HPA-axis regulation in the CNS for IL-1β is the hypothalamus, (reviewed in Weigent and Blalock (1995)). Furthermore, IL-1β is induced in the anterior pituitary via LPS administration and it is possible that IL-1β exhibits autocrine and paracrine regulation of the pituitary gland during infection (Koenig *et al.*, 1990).

IL-1β has been shown to stimulate another site within the HPA-axis, namely the adrenal cortex, to produce prostaglandins which eventually promote corticosterone release (Eskay *et al.*, 1990). IL-1β itself has been located in the adrenal gland, specifically in adrenal chromaffin cells and the adrenal cortex (Bartfai *et al.*, 1990, Scultzberg *et al.*, 1995). We now move on to the examination of IL-6 as a role player in stress.

IL-6, like IL-1β, is produced by both immune and non-immune cells. IL-6 producing cells in the neuroendocrine and endocrine tissues reside in the hypothalamus, the anterior pituitary and the adrenal cortex (Ohmichi *et al.*, 1992, Path *et al.*, 2000).

Proof of IL-6 being released from the adrenal cortex stems from studies done by Judd *et al.* (1990-1992) finding immunodetectable accumulation of IL-6 in the supernatants of rat *zona glomerulosa* cells after IL-1β and ACTH stimulation (Judd and Macleod, 1992, 1991, Judd *et al.*, 1990).

IL-6 expression rises in the midbrain after rats have been exposed to restraint or immobilization stress (Lozovaya and Miller, 2003). Furthermore, is evident that there is a rise in plasma IL-6 levels after non-inflammatory or non-infectious stress exposure such as exposure to a novel environment (Kronfol and Remick, 2000), electrical footshock (Zhou *et al.*, 1993), physical restraint (Nukina *et al.*, 1998a, Takaki *et al.*, 1994, Zhou *et al.*, 1993), exposure to open field (LeMay *et al.*, 1990), or conditioned aversive stimuli (Imura *et al.*, 1991, Kronfol and Remick, 2000). The increase in plasma IL-6 during conditions of psychological stress in rats occurs within 15 minutes which is much more rapid than when either local (turpentine) or systemic (LPS) inflammations are present, most likely due to catecholamine action (Tataki *et al.*, 1994, Turnbull and Rivier, 1999).

The involvement of peripheral catecholamines in elevating plasma IL-6 in immobilization stress seems to be independent of HPA-axis activation (Takaki *et al.*, 1994). Indeed, the role of the adrenal gland during psychological stress proves it to be most likely the biggest source of peripheral IL-6 (Zhou *et al.*, 1993). In restraint models of stress, the liver (and not the intestinal microflora as previously thought) is also considered to be one of the largest sources of plasma IL-6 (Nukina *et al.*, 2001).

However, in another study, elevated plasma IL-6 levels during immobilization stress have been found to be a result of both 1) CRH in the brain enhancing the activity of central catecholaminergic neurons and 2) activation of the peripheral sympathetic

nervous system (Ando *et al.*, 1998) by catecholamines released from the adrenal medulla and sympathetic nerve terminals, signalling increased plasma IL-6 levels (Tataki *et al.*, 1994). Taken together, these findings indicate that psychological stress induces IL-6 release *via* the sympathetic nervous system and the HPA-axis (pituitary and adrenal gland), but not directly from immune cells as suggested in a previous study (Zhou *et al.*, 1993).

IL-6 plays a role with regard to activating the HPA-axis directly (Mastorakos *et al.*, 1993). More specifically, IL-6 acts on the pituitary and adrenal gland, promoting CRH and AVP release, followed by ACTH and corticosterone secretion. Interestingly, the IL-6 produced in the adrenal gland is not sensitive to GC inhibition and can be released by IL-1β from this zone (Judd *et al.*, 1990). Intravenous injection of IL-6 causes a rise in rat plasma ACTH but to a lesser extend than the ACTH peak post IL-1β injection (Imura *et al.*, 1991). However, the response to IL-6 administration does not display a physiological indication of IL-6 regulation.

It is important to note that a discrepancy exists between humans and rats as far as the site of IL-6 mRNA expression (within specific cell types) in the adrenal gland are concerned. In humans, by combining immunohistochemistry with *in situ* hybridization, a study yielded the observation that most of the IL-6 mRNA signals were in cortical steroid producing cells in the inner cortical zones, islets in the medulla and macrophages, but no chromaffin cells (González-Hernández *et al.*, 1994). In rats on the other hand, most IL-6 mRNA signals were located in the medulla and only minor signals in the cortex (Gadient *et al.*, 1995). However, both these studies are relatively outdated, performed with older technology and reagents. Therefore, to probe the role of IL-6 in the stress response, all zones of the adrenal gland should ideally be assessed.

Under chronic stress conditions, IL-6 seems to cease acting on the pituitary gland, but continues to have an effect on the adrenals directly (zone non-specifically) to secrete GCs, possibly by autocrine mechanisms (John and Buckingham, 2003, Spath-Schwalbe *et al.*, 1994). With chronic stress, IL-1β may induce corticosteroid biosynthesis, independently of ACTH, and IL-6 stimulated corticosterone release from adrenocortical cells alone (John and Buckingham, 2003, Turnbull and Rivier, 1995). Under acute stress situations however, it has been postulated that IL-6 regulates the acute activation of the HPA-axis by exerting its effects on the hypothalamus and/or pituitary (John and Buckingham, 2003). The mechanisms underlying these discrepancies in HPA-axis regulation under acute *versus* chronic stress conditions, are apparent in the following paragraphs.

Acute stress activates the sympathetic nervous system and HPA-axis, additionally to parts of the immune system such as the increase of B-cells, natural killer cells and plasma IL-1β and IL-6 levels (Abraham, 1991, Maier and Watkins, 1998). Studies have found that hypothalamic IL-1β (Minami *et al.*, 1991) and mRNA (Shintani *et al.*, 1995) increase within 30 minutes after initiating immobilization stress and was still elevated at 120 minutes, or 60 minutes after the end of stress exposure. There are also elevations in plasma IL-6 levels within 15 minutes after the onset of acute stress but this rise is only modest and even though blood IL-6 elevations occur rapidly, it still lags behind that of ACTH, suggesting that IL-6 does not directly contribute to HPA function during acute stress (Zhou *et al.*, 1993).

These observations seem to be contradictory since it is known that IL-1β leads to IL-6 release. However, different cytokines may function at different absolute concentrations. Furthermore, the timing of sample collection might have influenced the results in the latter study. In light of our interpretation, it is incorrect to dismiss IL-

6 as a role player as presented by Zhou *et al*, (1993). Rather, the exact role of IL-6 should be more comprehensively investigated, keeping these possible confounders in mind.

Chronic psychological stress within an animal model ranges anything from 7 (Aguilera *et al.*, 1996, Banks *et al.*, 1995) days to 2 months (Hu *et al.*, 2000), with habituation taking place within three days in rats (Wilson, 2005). The traditional view held on the relations among chronic stress, depression and immunity is slowly starting to shift toward the notion that chronic stress and depression may actually enhance certain immune responses such as inflammation *via* an increase in IL-6 production (Robles *et al.*, 2005), although this is not a desired clinical outcome, given the extent that specific diseases, such as cancer, diabetes and heart disease, as well as psychiatric ill health, can be initiated or amplified by stress. The latter view suggests that a role exists for IL-6 in the stress response and that communication between IL-6 and GC control occurs.

Within the acute stress realm, GCs keep inflammatory responses in check by reducing the synthesis of proinflammatory cytokines. This defence mechanism is being overridden under conditions of depression and chronic stress whereby GC signals are disrupted. This leads to an overproduction of proinflammatory cytokines which in turn impairs corticosterone signalling by acting on GRs in the brain (Robles *et al.*, 2005). Under these circumstances of chronic stress, GR expression and GC binding capacity has been shown to decrease, which may imply a mechanism to reduce prolonged GC action (Al-Mohaisen *et al.*, 2000, Alexandrova and Farkas, 1992, Nishimura *et al.*, 2004).

In summary, IL-1β and IL-6 are produced and exert their effects at all three levels of the HPA-axis during psychological stress. However, differential regulation of these cytokines occurs under conditions of acute *versus* chronic stress. The dissociation seems to be at the level of the adrenal where chronic stress is implicated, unlike acute stress acting *via* all three levels of the HPA-axis. Also, GC feedback via GRs seems to be differently regulated during acute *versus* chronic stress.

2.3 Communication between GCs and cytokines

A number of disease states and pathologies are the result of degradation of the HPA negative feedback loop. Under ordinary conditions, negative feedback takes place by GCs binding with either GR or mineralocorticoid receptors (MR), primarily in the hippocampus. However, studies have indicated that loss of negative feedback control occurs under intensive acute stress or chronic stress conditions, with significant downregulation of both MR and GR mRNA levels in the hippocampus (Jacobson and Sapolsky, 1991). GC negative feedback control is also impaired by IL-1β and possibly IL-6 which affect MR affinity for GCs and promote stress hormone secretion (Lozovaya and Miller, 2003).

It has been proposed that a feedback loop exists between cytokines produced in the periphery by immune cells and the CNS (Licinio and Frost, 2000). For instance, intracerebroventricular administration of IL-1β has been shown to release IL-6 from the brain directly into the blood, without any CRH or peripheral sympathetic stimulus (Reichlin, 1993, Reyes and Coe, 1998b).

The effect that these cytokines have on the HPA-axis is exacerbated when both IL-1β and IL-6 or IL-1β and TNF-α are synergistically present. Of the three cytokines, it seems that IL-1β is solely implicated in the monoaminergic effects of a stressor, with

IL-6 and TNF-α affecting central monoamine activity to a lesser extent (Anisman, 2008). A bidirectional interaction exists between IL-1β and the HPA-axis, as IL-1β activates the HPA-axis and GCs suppress the production of IL-1β by decreasing IL-1β mRNA levels, by blocking post-transcriptional IL-1β synthesis *via* cAMP and by decreased release of IL-1β into the blood circulation (Lee *et al.*, 1988, Nguyen *et al.*, 2000). IL-1β also serves as an early cytokine in the cytokine cascade to increase downstream IL-6 and TNF-α production and feeds back on its original cellular sources (Kronfol and Remick, 2000, Shaftel *et al.*, 2008). It has been suggested that IL-1β-induced circulating IL-6 mediates HPA-axis responses to locally increased IL-1β levels (Shalaby *et al.*, 1989, Tosato and Jones, 1990). These cascades exhibit feedback loops, both positive and negative, and at different levels of the pathway. However, a complete, defined map of cytokine pathways and their receptors in the brain has not been elucidated.

Although not directly applicable, as far as the interaction between IL-6 and GCs are concerned, many studies have shown LPS-induced IL-6 plasma levels to be inhibited *via* the action of corticosteroids (Coelho *et al.*, 1995, Munck and Naray-Fejes-Toth, 1994, Schobitz *et al.*, 1993). However, few studies have revealed whether elevated plasma GC actually influence cytokine levels within the CNS. GCs have failed to inhibit central release of IL-1β-induced IL-6 into CSF following psychological stress (social isolation) in monkeys (Reyes and Coe, 1998b). The IL-6 in CSF was shown to be brain derived and not a result of passive diffusion from the blood into CSF (Reyes and Coe, 1998a).

The scenario where GCs inhibit IL-6 peripherally but not centrally may be explained by the different cell types releasing IL-6 into the CSF (astroglia, microglia, and neurons producing IL-6) *versus* the peripheral circulation (Kupffer cells of the liver

producing IL-6 most readily) (Joseph *et al.*, 1993, Liao *et al.*, 1995, Ringheim *et al.*, 1995) although the adrenal gland has also been pointed out as one of the most important sources of IL-6 (Zhou *et al.*, 1996). In other words, plasma GCs have more extensive access to cell sources of IL-6 located in the periphery, than in the brain (in order to exert its inhibitory effect). Taken together, it can be assumed that the increased release of IL-6 during stress is not under the inhibitory control of GC, probably because GCs are mainly released from the adrenal gland (Waage *et al.*, 1990), with limited access to central regulation of IL-6. The reverse (GC regulation by IL-6) has also been explored.

Previous work by our group investigated the role of IL-6 in the maintenance of IL-1β and corticosterone levels, and found that blocking IL-6 in effect dampened the secretion of corticosterone after repeated restraint stress (Smith *et al.*, 2006). This observation is supported by the notion that in conditions of prolonged stress and inhibition of CRH and ACTH by negative feedback of circulating GCs, IL-6 is responsible for maintaining elevated GC levels by acting on the adrenal gland to release GC (Path *et al.*, 2000). A second mechanism for sustaining GC levels is by means of IL-6 enhancing GC action by limiting downregulation of GR concentrations (Smith *et al.*, 2006).

In conclusion, it has been confirmed that IL-1β promotes IL-6 release, which in turn stimulates GC action. However, the release of these cytokines is inhibited indirectly *via* GC-induced negative feedback of the HPA-axis, and directly by GC inhibition of cells secreting these cytokines in the periphery, although diminished feedback occurs during chronic stress conditions.

2.4 Importance of receptors

To investigate the role of IL-1β, IL-6, GCs and GABA without measuring co-expressed levels of their respective receptors proves to be futile, as elevated protein levels do not always correspond with increased action of the particular protein. Therefore accurate conclusions cannot be drawn when receptor quantification is excluded. Measuring GR level of expression is of specific importance as GR occupation is required for GC negative feedback and subsequent control of the HPA-axis. Local negative feedback of the abovementioned mediators at the level of cells and tissues also employs receptor dynamics.

2.4.1 GABA receptors

GABA is an amino acid which is the major inhibitory neurotransmitter of the CNS, acting *via* opening of Chlorine channels which causes hyperpolarisation of GABA's postsynaptic target, leading to a reduced likelihood of firing an action potential (Sherwood, 2004).

Psychogenic stressors have been shown to regulate GABAergic neurons of the basal forebrain and hypothalamus (Herman *et al.*, 2004). GABA acts indirectly on the pituitary gland *via* the hypothalamus (Schimchowitsch *et al.*, 1991, Vincent *et al.*, 1982), and directly by being produced within the gland itself in an autocrine fashion, as investigated in rats and rhesus monkeys (Duvilanski *et al.*, 2000, Mayerhofer *et al.*, 2001). As nearly half of all synapses in the mpPVN and the majority of local inputs to this structure are indentified as GABAergic, GABA seems to be the main neurotransmitter involved in the regulation of CRH neurons (which express $GABA_A$ receptors) in the hypothalamus (reviewed in De Souza and Franci (2008)).

GABA has been shown to participate in the pathophysiology of affective disorders, in the development of certain types of behaviour and in neuronal regulation in the brain (reviewed in Otero Losada (1988)). However, the effect of stress on GABA regulation seems to be site specific, for example, GABA levels have been found to decrease with acute immobilization stress of one hour in the corpus striatum and to decrease in the frontal cerebral cortex after repeated immobilization stress of 30 minutes per day for 14 days (Otero Losada, 1988). Also, footshock has been shown to decrease GABA receptor binding in the CNS (Biggio *et al.*, 1981). Conversely, acute restraint stress has been found to increase GABA efflux region-specifically in the basolateral amygdala (Resnikov *et al.*, 2008). Acute swim stress has increased the density of high and low affinity binding sites for GABA in the mouse brain, but not with repeated stress exposures (Skerritt *et al.*, 1981). Also, another study demonstrated GABA to increase under conditions of cold and immobilization stress in the striatum and hypothalamus (Yoneda *et al.*, 1983). The effect of immobilization stress on GABA or GABA-R is thus equally unclear.

Three classes of GABA receptors exist, namely, $GABA_A$ (with subunits α1−6, β1−3, γ 1−3, δ, ε, π and θ) and $GABA_C$ (with subunits ρ1−3) ligand-gated chloride ion gated channels, and G protein-coupled $GABA_B$ receptors (Zemkova *et al.*, 2008). All three classes of receptors are expressed in the pituitary gland (Anderson and Mitchell, 1986, Boue-Grabot *et al.*, 2000), with $GABA_A$ (responsible for most of the actions of GABA in the brain) and $GABA_B$ receptors specifically expressed in the anterior pituitary (Mayerhofer *et al.*, 2001). A recent study showed that of all the $GABA_A$ subunits, α1 and β1 subunit proteins are present in the secretory anterior pituitary cells and that $GABA_A$ receptors function mostly to depolarise the cell wall, causing the activation of voltage-gated Ca^{2+} ion influx (Zemkova *et al.*, 2008).

Experimental animal models have demonstrated that GABA inhibits the CRH neurons *via* the $GABA_A$ receptor in the PVN of the hypothalamus under tonic conditions (Cole and Sawchenko, 2002, Herman *et al.*, 2003, Kovacs *et al.*, 2004, Mikkelsen *et al.*, 2008). $GABA_A$ receptor itself was down-regulated after one 3-hour exposure to immobilization stress (Zhang *et al.*, 1990). Stress has also been shown to decrease the function of the $GABA_A$ receptor complex (Biggio *et al.*, 1990).

An investigation regarding the regulation of GABA and IL-6 in relation to each other has found that intracerebroventricular injection of $GABA_A$ and $GABA_B$ receptor agonists inhibited restraint (1 hour) stress-induced increases in plasma IL-6 levels, whereas injection of an antagonist increased basal and restraint stress-induced plasma IL-6 concentrations (Song *et al.*, 1998). Also, tonic levels of both IL-6 and TNF-α were found to be inhibited by GABA involving the $GABA_A$ receptor (Song *et al.*, 1998) and possibly *via* the suppression of p38 activity (Spangelo *et al.*, 2004).

Support for bi-directional communication between GABA and IL-6 exists: IL-6 has been shown to stimulate GABA release from both the hypothalamus and posterior pituitary gland after depolarisation of the tissue, possibly *via* prostaglandins, but not under basal conditions (De Laurentiis *et al.*, 2000). With regard to the effect of GABA on corticosterone secretion, a recent investigation showed that the blocking of GABA receptors increases corticosterone secretion in response to ether-induced stress (De Souza and Franci, 2008). However, the investigations of the complex interactions between these parameters are preliminary and much is still unknown.

2.4.2 Corticosterone receptors in periphery and brain.

There are two classes of GRs, namely the Type 1, high affinity MR that mediate circadian GC rhythms and generally act to stimulate a response and Type 2, low affinity GR which mediate GC levels during stress and are inhibitory in some systems and excitatory in others (O'Connor *et al.*, 2000). An illustration of the opposing actions of GR can be obtained from a classification system based on two classes of GC performance that has been devised by Sapolsky and his group (Sapolsky *et al.*, 2000). These are firstly **modulating actions** which can be further subdivided into 1) **permissive GC actions**, set in place under basal conditions, priming the host defence mechanisms to possible stressful insults, *via* basal levels of GR; 2) **suppressive GC actions**, depicted by a rise in GR levels an hour or more after the onset of stress, preventing stress-induced cytokine actions from overshooting and 3) **stimulating GC actions** which mirror suppressive GC actions in terms of the timing of GR expression but unlike suppressive actions, stimulating actions enhance the activity of catecholamines and resulting cytokine induction, in effect mediating the SNS response to stress. The second class of GC actions are called **preparative actions** which modulate adaptation to stress, possibly *via* modifying gene expression.

For the purpose of this thesis, we will limit ourselves to the discussion of GRs only. GRs are expressed in most tissues although the density thereof may vary under different physiological conditions (Okret *et al.*, 1991) and the liver is thought of as the major metabolic target tissue for GCs (Al-Mohaisen *et al.*, 2000). Cytoplasmic GR contain three functional domains: the first is a carboxyterminal-ligand binding domain which binds GC, leading to the dissociation of the second domain, a heat shock protein (Lozovaya and Miller, 2003). The absence of this protein allows the GR to

translocate to the nucleus where a third domain, its midregion, binds to GC response elements on target genes, resulting in activation *via* the amino terminal sequence (O'Connor *et al.*, 2000).

Binding of GC to GR in the anterior pituitary gland, hypothalamus, basolateral amygdala, prefrontal cortex and the hippocampus results in inhibition of the secretion of ACTH and down stream regulators of the HPA-axis (Buckingham *et al.*, 1996, Furay *et al.*, 2008, Turnbull and Rivier, 1999). GC feedback occurs firstly at the pituitary, with rapid (within minutes) transcription-independent regulation of expression of POMC, ACTH, and CRH receptors (Hinz and Hirschelmann, 2000, Makino *et al.*, 1995). Feedback also occurs at the hypothalamus, resulting in regulation in the expression of CRH and AVP in the CRH neurons of the hypothalamus as well as GABAergic inhibitory synaptic inputs to these CRH neurons (Cullinan and Wolfe, 2000, Verkuyl *et al.*, 2004, Verkuyl *et al.*, 2005), although a differential regulation of GABA inputs under conditions of acute *versus* chronic GC elevation has been proposed (Verkuyl *et al.*, 2005).

Verkuyl and his group suggested that during acute stress, the activity of CRH-producing cells in the PVN *via* GC is under the control of firstly limbic projections relayed *via* GABAergic interneurons, secondly by GC binding to GR in the hypothalamus, resulting in decreased GABAergic control of PVN neurons, and thirdly, by humeral feedback on CRH-producing cells. These modalities are most likely in balance during physiological conditions (and possibly acute stress). However, under the influence of unpredictable chronic, psychological stress, it was found that the normal relative contribution to HPA-axis activity of each of these mechanisms was disrupted, which led to less GABAergic inhibition of PVN neurons, resulting in less HPA-axis negative feedback (Verkuyl *et al.*, 2005).

A role for negative GC feedback to the HPA-axis via GR in the forebrain (the hippocampus in particular) is widely accepted (Jacobson and Sapolsky, 1991), but these inhibitory effects are stressor modality dependent (Furay *et al.*, 2008). A recent study found that the forebrain (prefrontal cortex, hippocampus, and basolateral amygdala) GR were responsible for GC feedback in mice under conditions of both mild (elevated plus maze) and robust (30 minute restraint stress) psychogenic stressors but not in the case of a systemic stressor (hypoxia) (Furay *et al.*, 2008). The changes in HPA-axis regulation seen with chronic stress (15 days) were mediated by mechanisms independent of forebrain GR (Furay *et al.*, 2008).

In addition to different sites of inhibition in the brain, three phases of inhibition exist: 1) rapid feedback which takes place within less than 15 minutes of GC release and may occupy some other means of action than binding to intracellular steroid receptors with subsequent gene modification 2) Early delayed feedback which develops within one to two hours following a stressful insult and continues for up to 24 hours, resulting in the suppression of protein second messengers which are responsible for ACTH release 3) Late-delayed feedback which occurs within 12-24 hours and is characterised by gene suppression of POMC, CRH, and AVP and (Buckingham *et al.*, 1992).

A take home message therefore is that inhibition of parts of the stress response *via* GR takes place in different tissues and consults different mechanisms depending on the nature and duration of the stress.

2.4.3 IL-1β receptors

The IL-1β receptor belongs to the immunoglobulin supergene family (Turnbull and Rivier, 1995). Both subunits of IL-1β act through the same cell surface receptors, namely IL-1RI (locating to almost all cells including glial cells and neurons throughout the rat brain, concentrated in the pituitary gland, dentate gyrus, hippocampus, and the hypothalamus) and IL-1RII (on the surface of immune cells) which lacks an intracellular domain and seems to lack significant physiological function (Anisman, 2008, Lozovaya and Miller, 2003, Shaftel *et al.*, 2008, Weigent and Blalock, 1995). It has been shown that CRH stimulates the expression of IL-1RI in the pituitary gland of mice under conditions of stress and inflammation (Laye *et al.*, 1994).

IL-1β soluble receptor acts as an antagonist, preventing IL-1β from binding with its membrane bound receptors (Turnbull and Rivier, 1999). Binding of IL-1β and not its endogenous antagonist IL-1ra, leads to the initiation of mitogen-activated protein (MAP) kinase pathways and a signal transduction pathway involving the phosphorylation and degradation of the endogenous IF –κβ, ultimately resulting in translocation of NF-κβ to specific target genes in the nucleus (Shaftel *et al.*, 2008).

2.4.4 IL-6 Receptors

IL-6Rα is part of the hematopoietic growth factor receptor family (Turnbull and Rivier, 1999). As is the case with IL-6 (mentioned earlier), IL-6R mRNA synthesis is more pronounced in the rat adrenal medulla compared with the cortex as opposed to humans where IL-6R is predominantly expressed in the *zona reticularis* and the inner *zona fasciculata* and to a lesser extent in the *zona glomerulosa* and in chromaffin cells of the medulla (Path *et al.*, 2000).

The mechanism of action of the IL-6 receptor involves a signal transduction (JAK/STAT) pathway to activate the ras/MAPK cascade. It employs the combination of IL-6, the ligand-binding IL-6 receptor α-chain (IL-6Rα) and the signal transducing β-chain (gp130). These three components oligomerize to form a complex of at least two of each component which then results in the signalling processes conducted by gp130 (Anisman, 2008, Lozovaya and Miller, 2003, Path *et al.*, 2000).

Normally the binding of a cytokine to its soluble receptor inhibits its activity by preventing the cytokine from binding to its membrane receptor (soluble IL-1β receptor is no exception). However, this is not the case with IL-6 as binding to its soluble receptor enhances its activity (Kronfol and Remick, 2000, Turnbull and Rivier, 1999).

2.5 Summary

Distinctions between psychological and systemic stressors can be made based on differences in the sensory organ in the brain initiating the stress response, on whether immune cells are involved and whether processing in limbic areas are required. The stress model employed in this thesis is psychogenic in nature and therefore has the following characteristics:

Upon the onset of stress, the stress stimulus is firstly relayed to the cerebral cortex after which it is passed on to the hypothalamus from where a signal is sent to the adrenal medulla to secrete catecholamines. These pathways constitute the SAM-axis and activation of this route also leads to increased cytokine expression in the adrenal gland.

Simultaneously, the stress stimulus is processed in the limbic system after which it is relayed to the PVN *via* GABAergic neurons. The PVN also receives input in the form of feedback from the periphery *via* circulating GCs and cytokines reaching the brain. IL-1β and its receptor as well as IL-1β-induced IL-6 and IL-6R expression are up-regulated in this region by means of catecholamine-induced CRH. Bidirectional communication between GABA and IL-6 also influences the regulation of the PVN. IL-1β stimulates CRH to be released from the PVN in order to release ACTH form the anterior pituitary where GABA, IL-1β and IL-6 expression is enhanced. Negative feedback to this structure *via* GC keeps these responses in check. Finally, ACTH reaching the adrenal cortex causes the release of GCs which act to inhibit local pro-inflammatory cytokine production as well as the HPA-axis *via* negative feedback at the hypothalamus and pituitary. Expression of IL-1β and IL-6 and their respective receptors also increase in the adrenal, with IL-1β-induced IL-6 maintaining GC

levels. However, the interactions of these parameters are complex, and investigations into lasting effects are complicated by their wide variety of potential sources and differences in the time courses over which they exert their effects. Therefore, it is not always possible to extrapolate lasting responses to stress from studies investigating acute time points only.

Furthermore the above, somewhat simplified view on psychological stress-related pathways is still preliminary in that not much is known about cross-talk between mediators of stress and interactions between the central and peripheral systems. This leaves room for investigation of specific psychological stress models in terms of basal *versus* stress conditions and peripheral *versus* central sensitivity to adaptation of the mentioned role players in the stress response.

2.6 Hypothesis and aims

We hypothesised that the HPA response to psychological stress is dependent on IL-6, and that although we do not expect to find detectable levels of IL-6 at time points 24 hours after stress, we will find IL-6 dependent effects at these time points, both centrally and peripherally.

Specific aims included:

a) To induce repeated mild psychological stress (restraint)
b) To determine lasting effects of stress on the HPA-response, specifically in terms of the pituitary and adrenal response, while blocking IL-6 using a daily anti-IL-6 antibody (injected intraperitoneally)
c) To assess various modulators of stress at time points 24 hours after the end of exposure to stress
d) To investigate possible adaptation to repeated stress over time in glands of the HPA axis

Chapter 3: Materials and methods

3.1 Study design

3.1.1 Experimental animals

In total, 120 male Wistar rats were selected for by weight (70g to 120g) before purchase from the University of Cape Town. Upon arrival the rats were caged five per cage (this ensured a variety of rat sizes in each cage). They were housed in a colony room at 21 °C, with the room ventilated at 10 changes/hour and animals were exposed to a 12 hour day-night cycle (lights on at 6:30 am), *via* artificial illumination. They received standard rat chow (supplied by the Medical Research Council animal unit in Parow) and water *ad libitum*.

Ethical approval was granted by the Animal Ethics Committee of Subcommittee B of Stellenbosch University. Rats were acclimatised, and handled and weighed on a daily basis during the morning for the entire housing period (in order to get familiar with the researcher), until they reached a weight of 350 to 400g. Three weeks before the onset of an experimental protocol, rats were handled by means of holding them in the position in which they would be injected. This measure was taken to ensure that animals became accustomed to the experimenters and experimental injection procedure, in order to control for any confounding factors. All interventions were performed during a 08:00 to 10:00 time slot to ensure comparable corticosterone levels from day to day and across all groups.

3.1.2 Experimental groups

Rats were divided into four groups:

- No Stress Placebo group (CP): receiving no stress and a saline injection (n=30)
- Stress plus Ab group (SAb): receiving an Anti-IL-6 antibody (Ab) injection and subjected to the stress paradigm (n=30)
- No Stress plus Ab group (CAb): receiving the Ab injection but not subjected to the stress intervention (n=30)
- Stress Placebo group (SP): receiving a saline injection as well as exposure to stress (n=30)

Each of these four groups was further subdivided into three subcategories, being a one-, two-, or three-day stress or control intervention, ultimately resulting in ten rats per subgroup. For the purpose of discussing these different repetitions of stress, I will refer to them as time point 1, 2, and 3.

3.2 Intervention protocols

Injections involved either 0.9% saline (placebo) or 100 µg lyophilized goat-derived anti-IL-6 Ab (first dissolved in 1ml of sterile PBS according to manufacturer's instructions, after which it was diluted in sterile saline to yield a final concentration of 2µg/ml). The Ab solution was stored at 4 ^{0}C for the duration of the study. Injections were administered ip. at a dosage of 1ml/kg bodyweight.

During the stress experiment, rats were exposed to a mild psychological stressor imposed by restraint for an hour per day, in translucent Perspex boxes (dimensions 7 cm x 8 cm x 15 cm, designed by local manufacturer), 30 minutes after having

received a saline or Ab injection. This stress model is a recognised one, and has been used successfully by our group in the past (Smith, 2004, Smith *et al.*, 2007). During the period of restraint the rats could only turn around with great difficulty, but respiration was not impaired. The stress intervention took place in a room separate from the procedure room so as to avoid rats being exposed to blood odours. For each day of restraint, all rats destined for the stress regime were restraint at one time (i.e. not in batches), and at the same time every day, throughout the protocol. All restraint boxes were cleaned after usage and in a manner similar to the cleaning of housing cages. Rats were euthanized 24 hours after the intervention had been terminated.

3.3 Sacrifice, sample collection and preparation

All rats were transported from their room to the weighing area, where they received a 0.9 ml pentobarbitone sodium (euthanase) injection. No more than five rats were sacrificed per day. Any unusual reactions by the rats were noted down immediately before and during euthanization to serve as possible explanatory indices when results were being analysed. Lack of consciousness was confirmed when no response was elicited in the rat after the foot had been pinched softly. Whole blood was collected from the right ventricle of the heart *via* puncture with a 20 gauge, 1.5 inch needle into a 5ml syringe and immediately transferred to heparinized vacuum tubes (Vacutainer, Beckton Dickinson). Blood was kept on ice (no longer than 2h) before being centrifuged for 15 min at 3000 g at 4°C after which the plasma was aliquotted into 1.5 ml eppendorffs and stored at -80°C for later batch analysis. The pituitary gland and both adrenal glands were collected, trimmed of any visible connective tissue where required, and placed in 10% formal-saline filled containers for one hour after which samples were transported to the laboratory to be weighed (a

sufficiently sensitive balance was not available in the animal house). Samples were then stored for three days at room temperature to allow adequate fixation, before being processed for histology.

3.4 Sample analysis

3.4.1 Multiplex assay

Commercially available Bio-Plex Cytokine Assay kits (171-305008, 171-000201, 171-203001, 171-203060, 171-K11070, Bio-Rad Laboratories, Inc.) were used for the determination of plasma IL-1β, IL-6 and TNF-α in accordance with manufacturer's instructions. We included the assessment of plasma TNF-α levels in order to control for possible presence of acute inflammation, as it has been shown that TNF-α is not induced by our model of stress (Nukina *et al.*, 1998b, O'Connor *et al.*, 2003, Smith *et al.*, 2007).

3.4.2 Corticosterone Enzymeimmunoassay

Plasma corticosterone levels were assessed using a commercially available enzyme-linked immunosorbent assay (ELISA/EIA) kit with internal controls (catalogue AC-14F1, Immunodiagnostic Systems Ltd).

3.4.3 Histology

Tissues were placed in embedding cassettes, processed and impregnated with paraffin wax (Histosec, Merck) using an automated tissue processor (TISSUE TEK II, model 4640B, Lab-Tek division, Miles Laboratories Inc., Naperville, IL). A rotary microtome (Reichert Jung, Heidelberg, Austria) was used to cut 5 μm cross-sections of all samples, irrespective of whether a hematoxylin and eosin (H & E) or immunohistochemical protocol was employed. The pituitary gland sections were

stained with H & E using standard protocols (in order to determine the location of the anterior pituitary). We decided to focus on the anterior (excluding the posterior) pituitary in our analysis as CRH-induced ACTH is released from this structure. Haematoxylin stains the basophilic structures (usually containing nucleic acids) blue-purple whereas the alcohol-based eosin stains the eosinophilic structures (intra-or extracellular proteins) bright pink. In particular, corticotrophs, a type of basophil which secretes ACTH, can be distinguished based on the affinity of the cells for the dye (H&E - see Appendix B for a detailed description).

3.4.4 Immunohistochemistry

Because stress exposure elevates cytokine protein independently of mRNA expression (caused by changes in protein expression due to differential regulation of translation, posttranslational processing or protein degradation) (Deak *et al.*, 2004, O'Connor *et al.*, 2003), analysis of cytokine protein levels instead of mRNA seemed more plausible in our investigation. We therefore included the investigation of relevant protein expression in the anterior pituitary and adrenal in the present study.

We verified that the anti-IL-6 Ab did indeed reach the pituitary gland and the adrenal glands as staining for the Ab itself yielded the fluorescence expression as depicted in Fig 2. Of interest, the availability of Ab differed between glands as well as between the different zones in the adrenal gland.

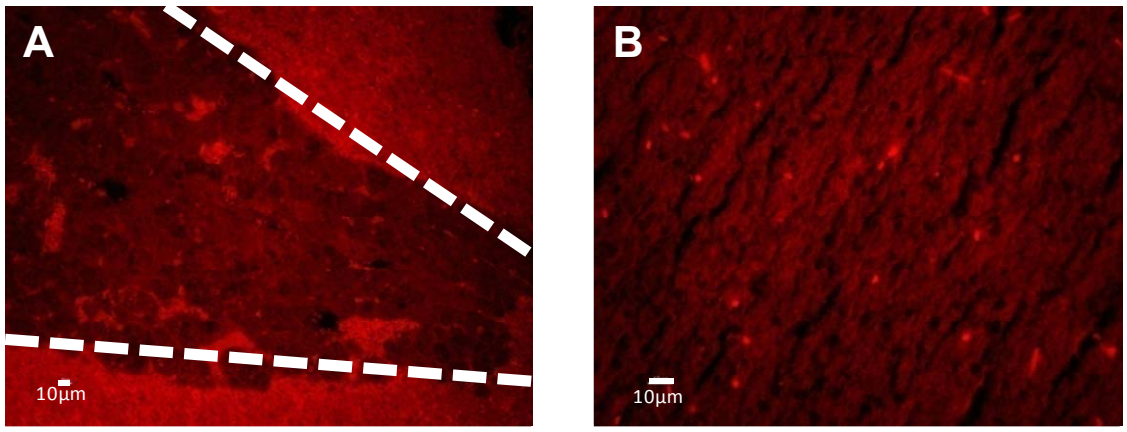


Figure 2: Anti-IL-6 Ab expression (red fluorescence) in (A) the adrenal where the medulla is enclosed by dashed lines and the reticularis the region surrounding it and (B) in the pituitary gland (images were taken at 40x magnification).

The antibodies used are summarised in Table 1. 0.1 M phosphate-buffered saline (PBS:1 ℓ of 1 M phosphate buffer, 90 g NaCl, 9 ℓ ddH_2O; pH 7.4) was used for all dilutions.

Table 1: Antibodies used to identify IL-6, IL-6R, IL-1β, IL-1βR, IL-1R, and GR in the pituitary and adrenal and $GABA_AR_{\alpha 1}$ expression in the pituitary gland.

Antibodies	**[Stock] (µg/ml)**	**Dilution**	**Catalogue # and supplier**
Primary antibodies:			
Goat polyclonal IL-6 (M-19)	200 µg/ml	1/200	sc-1205, Santa Cruz
Rabbit polyclonal IL6R-α (H-300)	200 µg/ml	1/100	sc-13947, Santa Cruz
Rabbit polyclonal IL-1β (H-153)	200 µg/ml	1/50	sc-7884, Santa Cruz
Rabit polyclonal IL-1R (H-150)	200 µg/ml	1/50	sc-25775, Santa Cruz
Rabbit polyclonal GR (H-300)	200 µg/ml	1/50	sc-8992, Santa Cruz
Goat polyclonal GABAARα1 (D-18)	200 µg/ml	1/200	sc-31404, Santa Cruz
Conjugated secondary antibodies:			
Donkey anti-rabbit (FITC) (D-1808)	200µg/0.5ml	1/200	sc-2090, Santa Cruz
Donkey anti-goat (Texas Red) (G-1708)	200µg/0.5ml	1/200	sc-2783, Santa Cruz

Each slide was coated with Poly-$_L$-Lysine (Sigma-Aldrich) and contained three sections per sample. Both adrenal- and pituitary gland samples were stained for the following markers: IL-6, IL-6R, IL-1, IL-1R, and GR, with $GABA_AR_{\alpha 1}$ additionally stained for only in the pituitary. We stained for the GABA receptor as the neurotransmitter itself has a fast phase half-life of 30 min to an hour and a slower phase exceeding one hour (Collins, 1972). Of these markers, only IL-1 and IL-1R were co-stained. Before the staining protocol was followed, slides were taken though a series of rehydration steps (see Appendix C). A brief outline of the staining procedure is as follows:

Slides were dried using a paper towel after which they were encircled with a wax pen. PBS was dropped onto the sections immediately after being dried (sections were kept under humidified conditions during the whole procedure). Sections were then incubated for 30 minutes in 5% donkey serum at room temperature in order to block non-specific binding sites. The serum was shaken off and primary Ab added in the pre-determined optimum concentrations after which it was left for four hours at room temperature or overnight at 4°C. Slides were washed three times with PBS before the secondary Ab (1/200) was added (donkey anti-rabbit in the case of IL-6, IL-6R, IL-1, and GR, and donkey anti-goat in the case of GABA and IL-1R). After an hour, Hoechst (1/200) was added for 15 minutes in order to stain for nuclei. In the case where markers were co-stained, two additional steps were included before adding Hoechst: 1) slides were washed and the second primary Ab was added for four hours at room temperature and 2) the secondary Ab was added for one hour after washing with PBS. Cover slips were mounted onto the slides by using fluorescent mounting medium (Dako, Diagnostech). See Appendix D for an elaboration on the protocol.

3.4.5 Image analysis

A negative control stain (PBS control) was performed with both secondary antibodies separately, as well as co-stained, in order to verify true positive staining. As absolute values were not taken into account in the quantification of the fluorescent images and merely differences between groups and zones within groups were of interest, image analysis was not corrected for in terms of the negative control. Furthermore, by not subtracting the negative control values from the positive results obtained, conservativeness of analysis was preserved. A high degree of confidence in positive staining was displayed in that all the data obtained were higher than the respective negative controls for the glands and zones.

Photos were taken: three fields of view per zone or pituitary gland (in the case of the adrenal gland; zones being the *glomerulosa*, *fasciculata*, *reticularis*, and *medulla*), per section (three sections per slide) of each of four slides per adrenal gland and five slides per pituitary gland for every marker across all four groups (1 time point only).

Photos were taken at 40x magnification using a microscope (Nikon ECLIPSE E400; 400x objective used), equipped with a colour digital camera (Nikon DXM1200) and a computer programme (*Simple PCI* version 4.0, Compix Inc., Imaging Systems, USA). Areas to be analysed were randomly selected from the central portion of each zone. With the adrenal gland slides, the appropriate zones were cropped to ensure exclusion of bordering zones from the photo. All photos were taken using identical filters, exposure times, and sensitivity. Each batch (taken over a period of at least a week) included samples from both the pituitary and adrenal and across all zones (see also Fig. 9). All photos were analyzed using the software package Image J version 1.41O (Rasband, 1997-2009). The Mean Gray Value (the sum of the gray

values of all the pixels in the selection divided by the number of pixels*)* for the images was automatically calculated and each pixel automatically converted to grayscale*,* using the following formula: gray = *(*red + green + blue*) /* 3*.* The fluorescence unit obtained refers to the relative area of the image that fluoresces, not to fluorescent intensity.

This measurement is fully automated and the software was obtained from the National Institute of Health (NIH), allowing one to assume that it is of sufficient quality. However, as is the case with all fluorescent analysis, this technique proves to exhibit a subjective component in that the researcher has to adjust image brightness and in this way decides subjectively as to what background *versus* positive staining is. Nevertheless, this is common practice today, and in the current study, all analysis were performed by one researcher (the candidate) only, thereby avoiding variation in the data due to researcher-specific differences in means of analysis.

3.5 Statistical analysis

The computer software Statistica version 7 (StatSoft Software) was used for all statistical analysis. Determination of effects of time, Ab treatment, stress, and adrenal gland zone specifications were analyzed using factorial analysis of variance (ANOVA) with Bonferonni and Fisher (LSD) *post hoc* tests. A p-value smaller than 0.05 was considered significant and results pertaining to masses were reported as means ± standard deviation (SD) and in relation to all other data as means ± standard error of the mean (SEM).

Chapter 4: Results

The results section is presented in three sections. Firstly, we present basic descriptive data to illustrate the efficacy of our model as a model for mild, psychological stress, as well as its sensitivity to probe the role of inflammatory mediators. Secondly, the effect of both stress and blocking of IL-6 on circulating cytokine levels over time is presented. Thirdly, we present data obtained at a time point 24 hours after a single exposure to stress, to illustrate lasting effects of the stress response in various tissues.

4.1 Stress model

We assessed both pituitary and adrenal mass as effector tissues of stress perception and HPA-axis activation respectively, with the assumption that a higher mass will indicate greater stress-related activity of the particular gland. Fig. 3 illustrates no significant effect of stress alone on pituitary mass. In controls, blocking IL-6 resulted in transient increase in pituitary mass after 2 stress exposures only. However, when blocking IL-6 in the presence of stress, this up-regulation, although again of transient nature, occurred after only 1 exposure to stress.

Similar to the result for pituitary mass, stress alone also seemed to have no effect on adrenal mass (Fig. 4). Blocking IL-6 resulted in a decrease in average adrenal mass, in a manner independent of stress. This was an effect that was only significant after one stress exposure, probably due to the fact that in CP rats, adrenal mass was significantly lower at time point 2, when compared to the same group at time points 1 and 3.

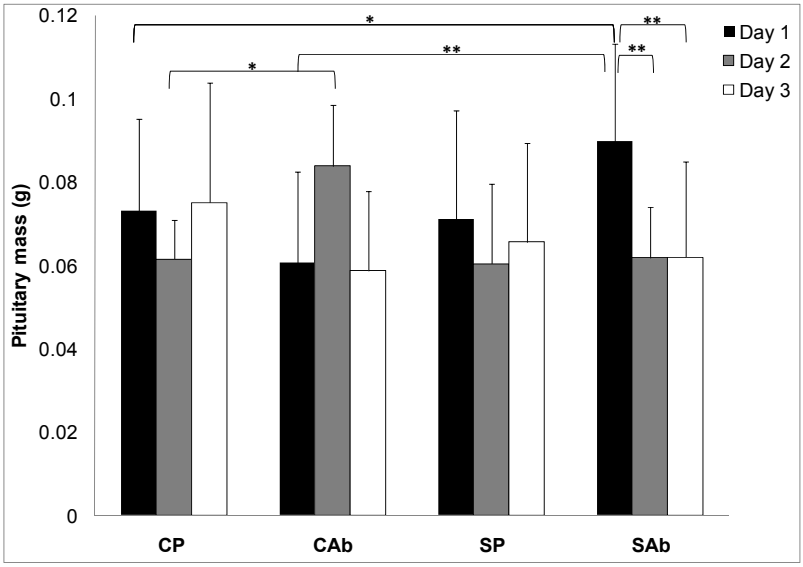


Figure 3: Pituitary mass. Results are expressed as means ± SD and were analysed using factorial analysis of variance (ANOVA) with Fisher (LSD) *post hoc* tests (* $p < 0.05$; ** $p < 0.005$). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab. n = 10 rats per time point per group.

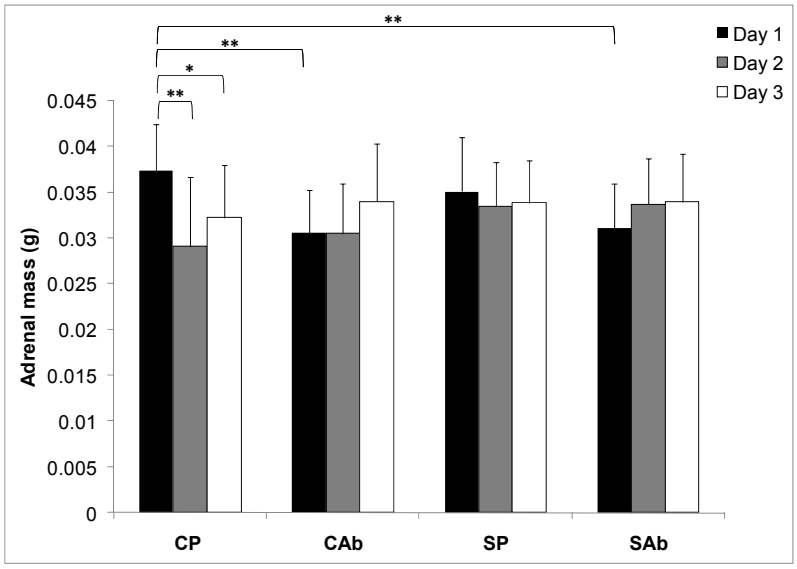


Figure 4: Adrenal mass. Results are expressed as means ± SD and were analysed using factorial analysis of variance (ANOVA) with Fisher (LSD) *post hoc* tests (* $p < 0.05$; ** $p < 0.005$). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab. n = 10 rats per time point per group.

Plasma corticosterone concentrations are presented in Fig. 5. When considering the data obtained after one placebo injection and stress exposure only, stress resulted in a significant increase in circulating corticosterone levels (CP: 47.9±13.2; SP: 132.7±43.4). This response was attenuated in the absence of IL-6 (SAb: 58.3±16.3). In unstressed animals, blocking IL-6 had no effect on this parameter. Due to high intra-group variation, no statistically supported conclusions could be drawn with respect to possible adaptations over time, although the effect of stress alone (SP) seemed to diminish over time.

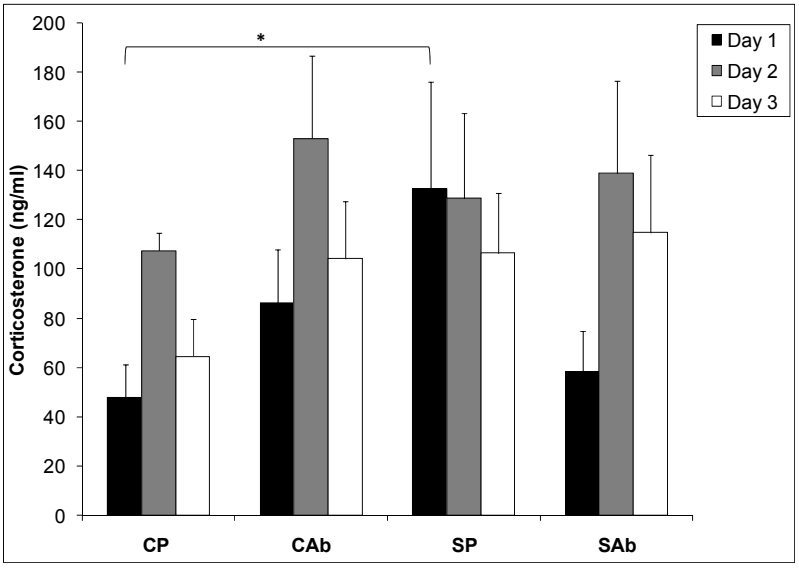


Figure 5: Plasma corticosterone concentrations. Results are expressed as means ± SEM and were analysed using factorial analysis of variance (ANOVA) with Fisher (LSD) *post hoc* tests (***** $p < 0.05$; ****** $p < 0.005$). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab. n = 10 rats per time point per group.

4.2 Effect of blocking IL-6 on circulating cytokine profile

Although we know that the IL-6 response (evident in the blood) to stress is transient and rather short-lived, and we therefore did not expect to see elevated levels at the time point of sacrifice, we included the IL-6 analysis only since an anti-IL-6 Ab was

used. As expected, blood IL-6 levels were almost non-detectable at all time points, with no apparent effect of stress, IL-6 Ab or time (Fig. 6). Due to technical difficulties, no data is available for time point 3.

IL-1β concentrations are represented in Fig. 7. At time point 1, blocking IL-6 decreased IL-1β levels, but only in the absence of stress. This effect was not evident at subsequent time points. Stress alone did not have any significant effect on IL-1β concentrations.

With regard to TNF-α (Fig. 8), neither stress, nor blocking of IL-6, had any significant effect. However, there was a similar stepwise decline in TNF-α concentration over time in all groups (ANOVA main effect of time: $P < 0.001$).

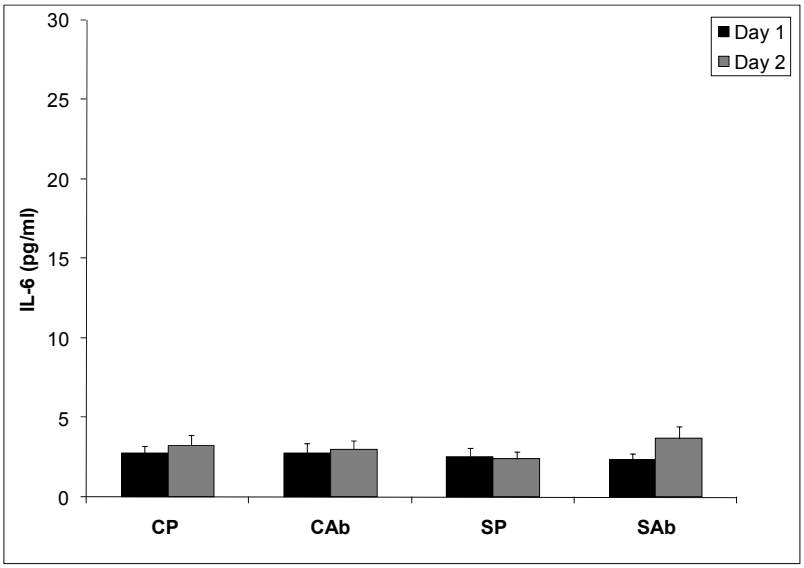


Figure 6: Plasma IL-6 concentrations. Results are expressed as means ± SEM and were analysed using factorial analysis of variance (ANOVA) with Fisher (LSD) *post hoc* tests. Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab. n = 10 rats per time point per group.

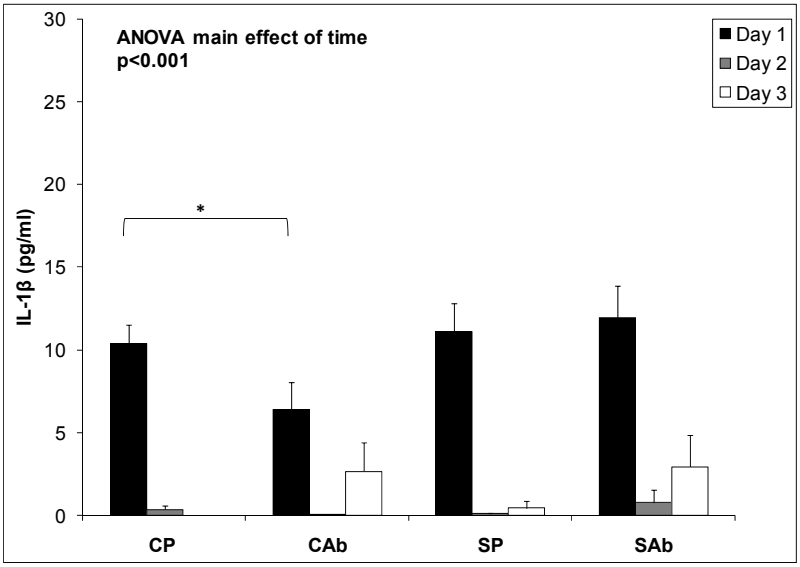


Figure 7: Plasma IL-1β concentrations. Results are expressed as means ± SEM and were analysed using factorial analysis of variance (ANOVA) with Fisher (LSD) *post hoc* tests (***** p < 0.05; ****** p < 0.005). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab. n = 10 rats per time point per group.

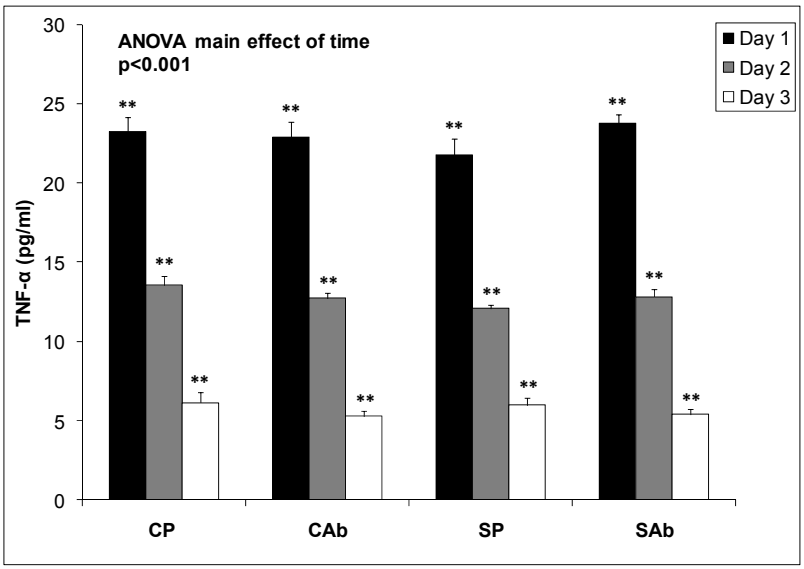
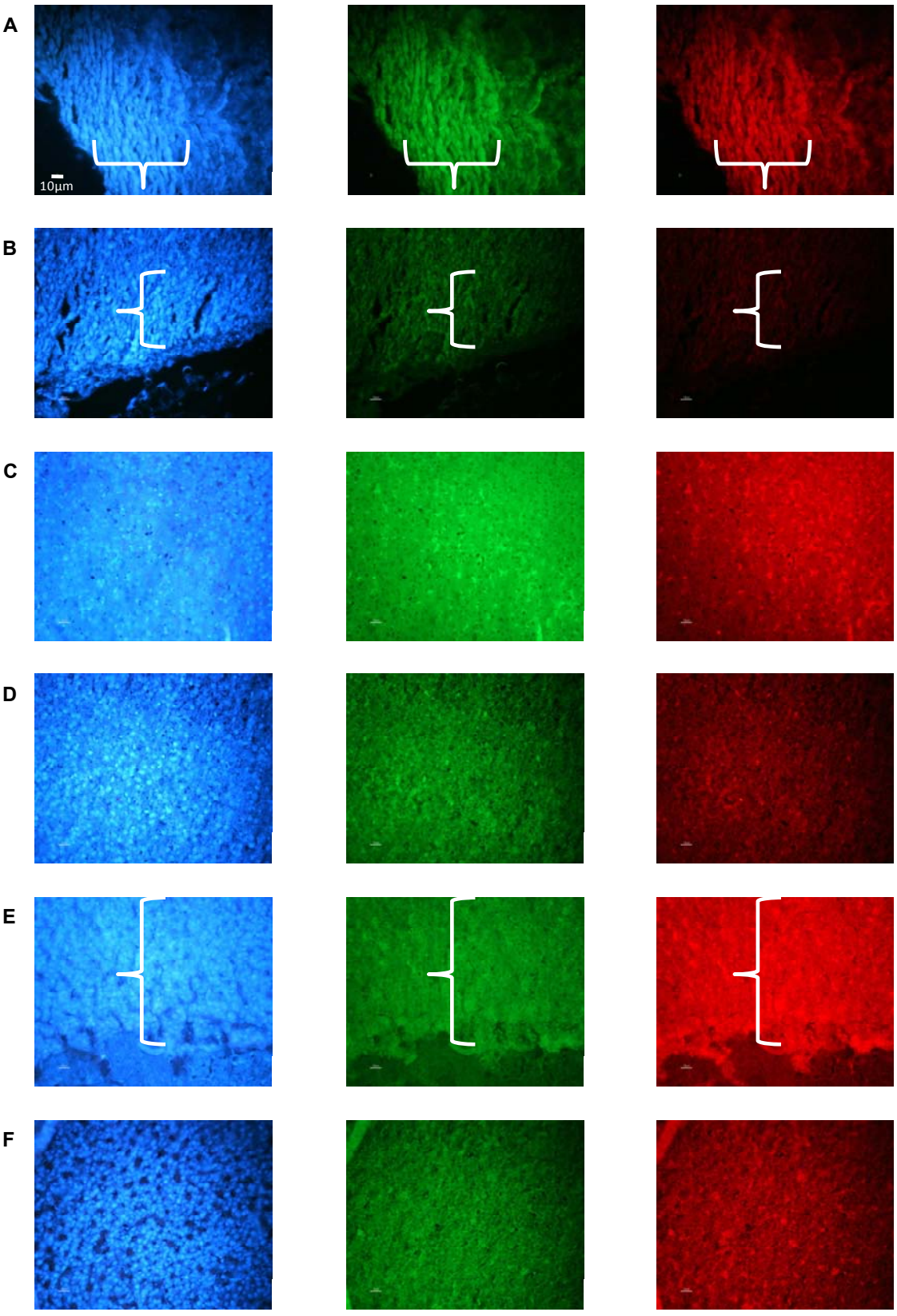


Figure 8: Plasma TNF-α concentrations. Results are expressed as means ± SEM and were analysed using factorial analysis of variance (ANOVA) with Fisher (LSD) *post hoc* tests (***** p < 0.05; ****** p < 0.005). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab. n = 10 rats per time point per group.

4.3 Lasting effects of stress at tissue level

Due to the presence of a possible confounder (refer to discussion section p65), assessment of lasting effects of stress at the tissue levels, in the pituitary and adrenal glands, were limited to time point 1 . All parameters assessed using fluorescent microscopy produced similar diffuse fluorescent staining. A representative collection of images of these results (limited to IL-1β in this case) is presented in Fig 9 on the following two pages.


A
B
C
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10µm

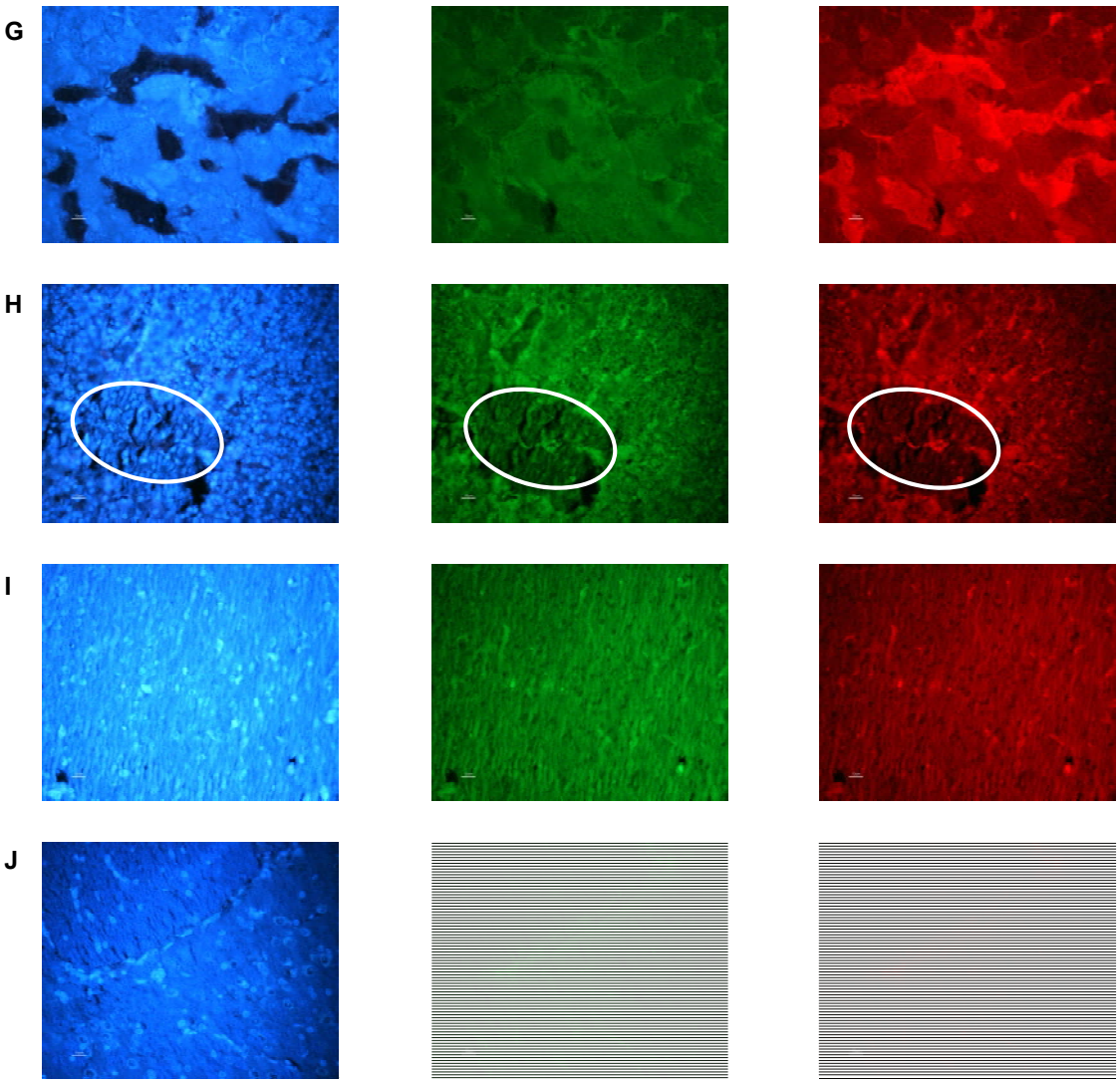

Figure 9: Representative images of fluorescent staining of adrenal and pituitary glands for nuclei (blue), IL-1β (green) and IL-1βR (red). Row **A** and **B**: adrenal *zona glomerulosa* (enclosed in white bracket) where row B = negative control; Row **C** and **D**: adrenal *fasciculata* where Row D = negative control; Row **E** (region inside bracket) and **F**: adrenal *reticularis* where row F = negative control; Row **G** and **H** (region inside boundary): adrenal medulla where Row H = negative control and Row **I** and **J**: pituitary where row J = negative control. Images were taken at 40x magnification.

Perception of stress alone did not seem to affect expression of $GABA_AR_{\alpha 1}$ in the anterior pituitary at the time point assessed (Fig. 10). Blocking IL-6 significantly increased $GABA_AR_{\alpha 1}$ expression in the absence of stress, but not in the presence of stress. A weak interaction effect of the Ab with stress was evident (Fig. 10 insert).

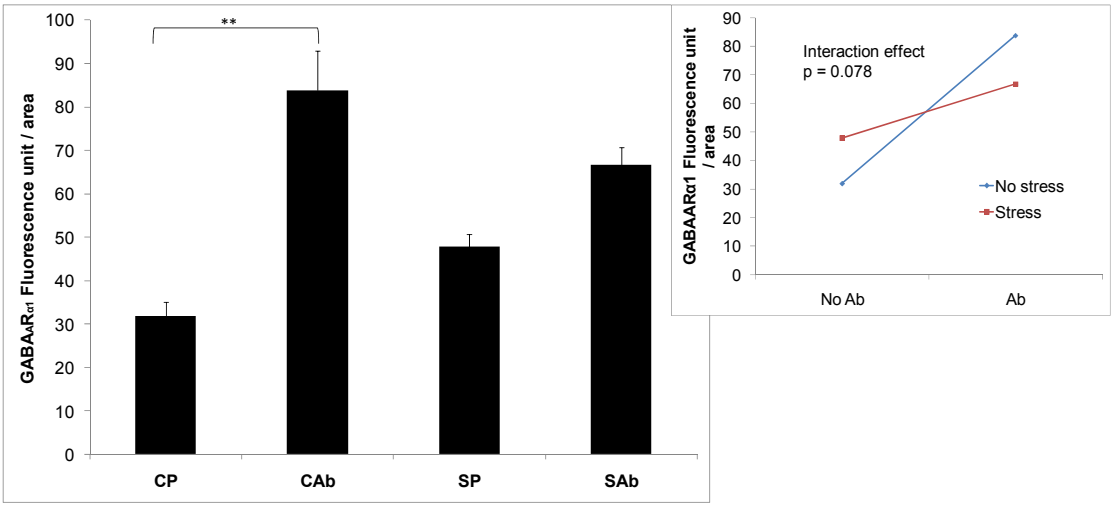

Figure 10: Pituitary $GABA_AR_{\alpha 1}$ expression. Results are expressed as means ± SEM and were analysed using factorial analysis of variance (ANOVA) with Bonferonni *post hoc* tests (***** $p < 0.05$; ****** $p < 0.005$). Factorial ANOVA indicated a tendency for an interaction effect of stress and Ab treatment (insert). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab n = 5 rats per group.

Pertaining to the pituitary gland, the only other significant increases from baseline were in the CAb groups for IL-1β and its receptor (Fig. 14 and 15). Results obtained for adrenal expression of GR (Fig. 11), as well as for IL-6, IL-6R, IL-1β and IL-1βR (Figs. 12-15), illustrated a consistent picture. For all parameters, statistical analysis indicated that expression levels were of the same order in the different zones of the adrenal gland (CP). Stress alone consistently up-regulated expression of all parameters assessed. This up-regulation was of similar magnitude in all zones of the adrenal – although up-regulation in the medulla seemed less pronounced. With regard to the effect of IL-6, blocking IL-6 in the absence of stress also up-regulated expression of all parameters (CP *vs*. CAb) (Fig.12). However, here the magnitude of up-regulation differed substantially between the different adrenal zones, with highest up-regulation in the *zona fasciculata* and the *zona reticularis*. When blocking IL-6 in the presence of stress, the combined up-regulatory effect of stress and IL-6 Ab was not additive, but the zone specific differences seen when blocking IL-6 in CAb, were still evident.

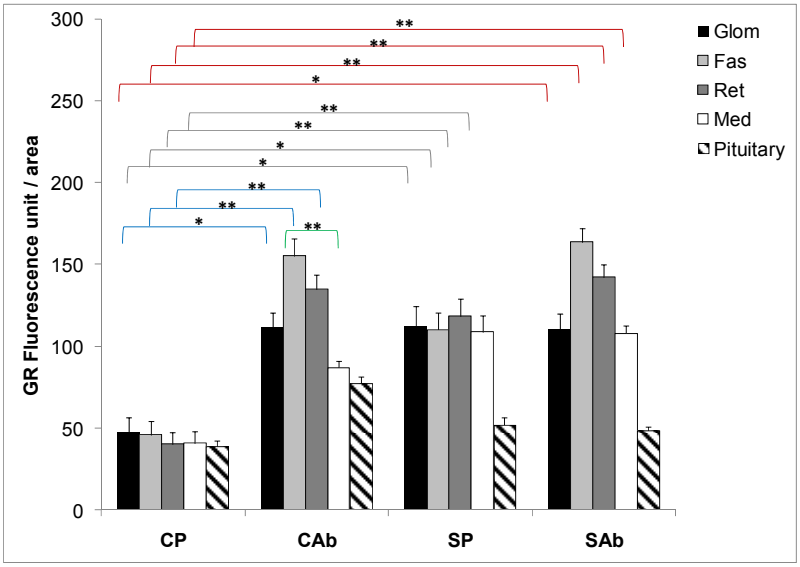

Figure 11: Tissue GR expression. Results are expressed as means ± SEM and were analysed using factorial analysis of variance (ANOVA) with Bonferonni *post hoc* tests (* $p < 0.05$; ** $p < 0.005$). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab; Glom, Glomerulosa; Fas, Fasciculata; Ret, Reticularis; Med, Medulla. n = 4 rats per adrenal zone per group and n = 5 rats per pituitary per group. Colour codes: Red, effect of stress and Ab; Grey, effect of stress; Blue, effect of Ab; Green, differences between zones.

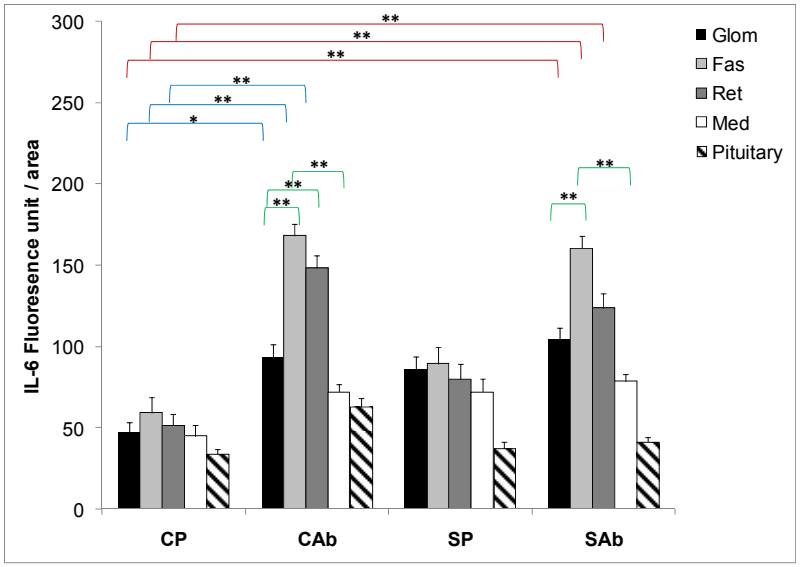

Figure 12: Tissue IL-6 expression. Results are expressed as means ± SEM and were analysed using factorial analysis of variance (ANOVA) with Bonferonni *post hoc* tests (* $p < 0.05$; ** $p < 0.005$). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab. Glom, Glomerulosa; Fas, Fasciculata; Ret, Reticularis; Med, Medulla. n = 4 rats per adrenal zone per group and n = 5 rats per pituitary per group. Colour codes: Red, effect of stress and Ab; Grey, effect of stress; Blue, effect of Ab; Green, differences between zones.

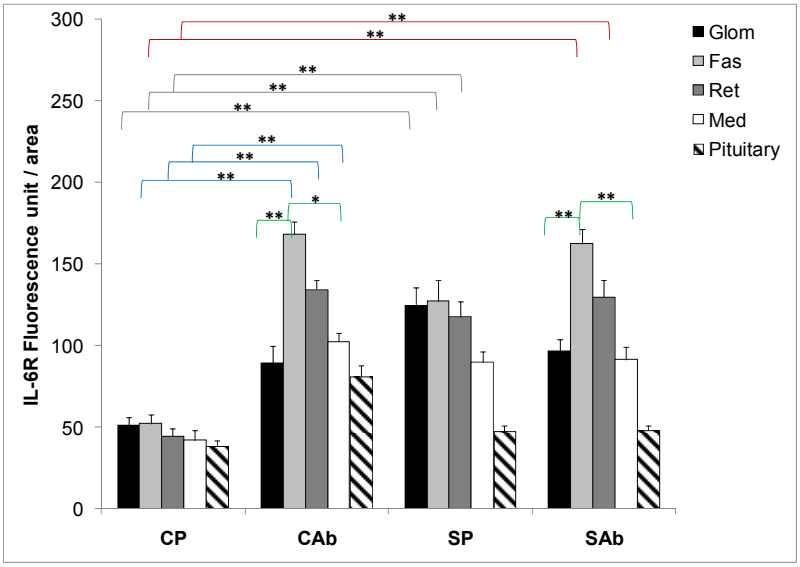

Figure 13: Tissue IL-6R expression. Results are expressed as means ± SEM and were analysed using factorial analysis of variance (ANOVA) with Bonferonni *post hoc* tests (* $p < 0.05$; ** $p < 0.005$). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab; Glom, Glomerulosa; Fas, Fasciculata; Ret, Reticularis; Med, Medulla. n = 4 rats per adrenal zone per group and n = 5 rats per pituitary per group. Colour codes: Red, effect of stress and Ab; Grey, effect of stress; Blue, effect of Ab; Green, differences between zones.

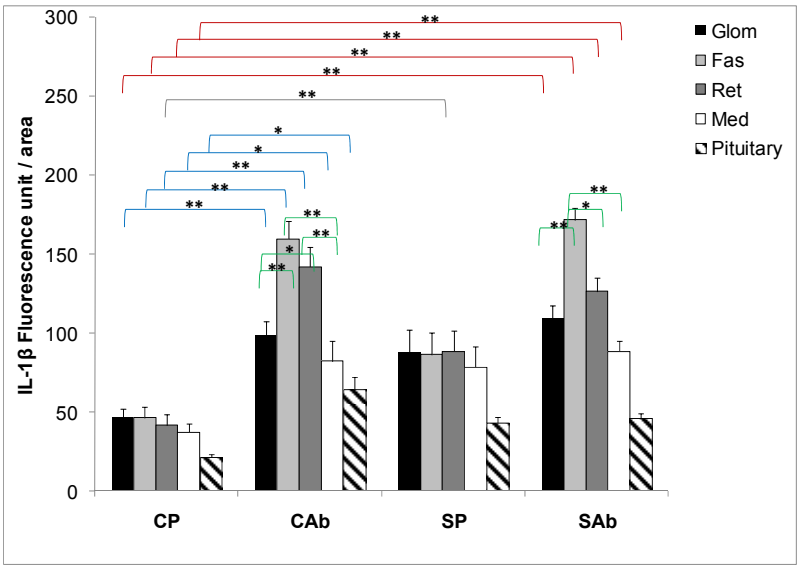

Figure 14: Tissue IL-1β expression. Results are expressed as means ± SEM and were analysed using factorial analysis of variance (ANOVA) with Bonferonni *post hoc* tests (* $p < 0.05$; ** $p < 0.005$). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab; Glom, Glomerulosa; Fas, Fasciculata; Ret, Reticularis; Med, Medulla. n = 4 rats per adrenal zone per group and n = 5 rats per pituitary per group. Colour codes: Red, effect of stress and Ab; Grey, effect of stress; Blue, effect of Ab; Green, differences between zones.

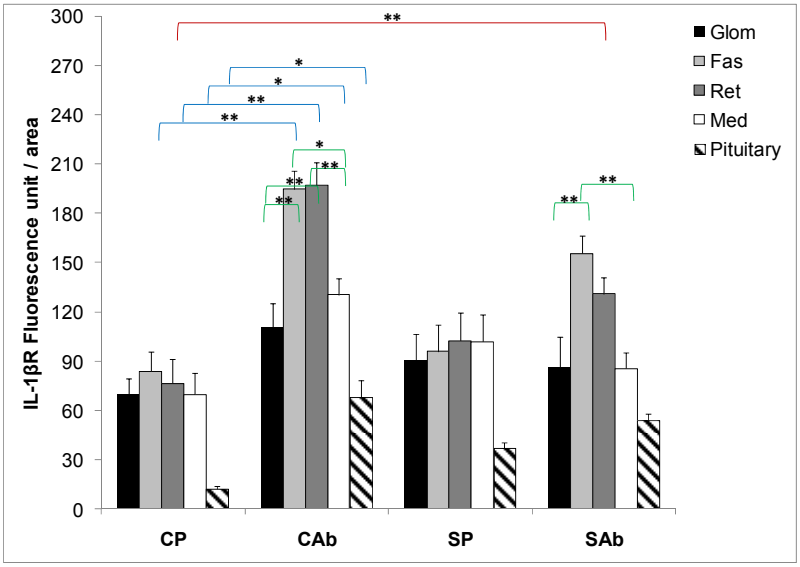

Figure 15: Tissue IL-1βR expression. Results are expressed as means ± SEM and were analysed using factorial analysis of variance (ANOVA) with Bonferonni *post hoc* tests (***** $p < 0.05$; ****** $p < 0.005$). Abbreviations: CP, Control Placebo; CAb, Control Ab; SP, Stress Placebo; SAb, Stress Ab; Glom, Glomerulosa; Fas, Fasciculata; Ret, Reticularis; Med, Medulla. n = 4 rats per adrenal zone per group and n = 5 rats per pituitary per group. Colour codes: Red, effect of stress and Ab; Grey, effect of stress; Blue, effect of Ab; Green, differences between zones.

Chapter 5: Discussion

We verified that that our model of restraint stress did indeed induce stress, as plasma corticosteroid levels remained significantly increased 24 hours after a single stress exposure. This result is consistent with other reports in the literature such as the increased corticosterone levels observed for 48-96 hours after a single exposure to inescapable shock (IS) (O'Conner *et al.*, 2003), as well as previous work by our group, using stress models of lesser severity (Smith *et al.*, 2007, Wilson, 2005).

Also similar to previous findings by our group (Smith *et al.*, 2007), this corticosterone response was blunted when blocking IL-6. Given the well-known fact that corticosterone is released as an anti-inflammatory reaction to pro-inflammatory stimuli, this blunted response is probably due to the net effect of lower IL-6 levels in circulation. However, since IL-6 was responsible for maintaining GC levels, our data also suggest that IL-6 is a vital stimulator of GC release from the adrenal gland *via* catecholamines or prostaglandins along with the signal provided *via* the classical activation of the HPA-axis (Path *et al.*, 2000, Takaki *et al.*, 1994).

Although the lasting corticosterone response to the first stress exposure was relatively clear, the response over time in terms of corticosterone and the role of IL-6, is more difficult to interpret, since no consistent pattern emerged. Of interest is the fact that mean corticosterone concentrations were relatively high in the control groups, when compared to previous studies by our group (20 ng/ml in Smith *et al*., 2007 *versus* 47.9 ng/ml in the present study). One possibility is that the animals mounted an additional local acute inflammatory response as a result of minor needle-induced tissue damage caused by the injection of placebo or Ab itself. This

idea is supported by the fact that TNF-α levels were significantly elevated across all experimental groups, when compared to earlier results by our group using both acute and chronic models of restraint, showing non-detectable TNF-α levels (Smith, 2004). This indicates that TNF-α levels are not affected by restraint as used in this particular model. This suggestion can be further strengthened by the literature that has found TNF-α to be more tightly associated with systemic stress than psychological challenge (Nukina *et al.*, 1998b, O'Conner *et al.*, 2003). Furthermore, TNF-α levels in the current study showed a stepwise decrease over time in all groups, suggesting an acute inflammatory response to the injection, to which the animals habituated over time. Given this confounder, the decrease in levels as investigated over time may not be a true reflection of adaptation. Therefore, although all results over time are presented in the result section, we will focus on the discussion of lasting effects measured at the time point 24 hours after the first exposure to stress.

In terms of other circulating cytokine concentrations, we report a) very low IL-6 levels across all groups, with no significant effect of prior stress or IL-6 Ab administration, and b) decreased IL-1β levels when IL-6 was blocked in unstressed animals. The very low levels of plasma IL-6 may be explained by the short half-life of IL-6 (3 minutes), which is common amongst other cytokines (Castell *et al.*, 1988, Vilcek, 2003) and the fact that we only measured IL-6 levels 24 hours after it had been released. Plasma IL-6 may very well have been transiently increased directly after the stress intervention and cleared by the time of sacrifice. From the literature, it is known that IL-6 is acutely released in response to restraint stress (Takaki *et al.*, 1994, Zhou *et al.*, 1993), although there is still controversy with regard to the relative contribution of central IL-6 production versus catecholamine-stimulated IL-6

production in elevating the circulating IL-6 levels observed (Ando *et al.*, 1998, Nukina *et al.*, 1998a, Takaki *et al.*, 1994). Therefore, when investigating lasting effects of an acute stressor, as in the current study, simultaneous assessment of IL-6 and IL-6 receptor expression at tissue level, both centrally and peripherally may provide more information (see later).

The fact that plasma IL-1β concentrations mirrored that of TNF-α in the sense that levels were significantly lower in day two and three than day one may demonstrate positive feedback of TNF-α on IL-1β secretion, as seen in classical models of inflammation (Ebisui *et al.*, 1994). However, although TNF-α may have had a confounding effect on the IL-1β levels, we observed significant differences between groups with regard to the latter cytokine at the first time point. We therefore assume that these differences observed were the effect of the particular interventions implemented in the study. The fact that plasma IL-1β concentrations were decreased after blocking IL-6 in unstressed animals (CAb), suggest that IL-6 has a role in the maintenance of IL-1β levels under basal physiological conditions, as was also illustrated by an earlier study by our group (Smith *et al.*, 2007). Although it has been established that IL-6 negatively regulates IL-1β levels in a stressed condition (Nukina *et al.*, 2001, Schindler *et al.*, 1990), our group is the first to present data suggesting the reverse in absence of stress. This IL-6-induced IL-1β scenario was no longer evident in a stressed condition, possibly due to a relatively diminished anti-inflammatory capacity, resulting from the lower corticosterone response seen in SAb, in combination with a transient increase in IL-1β levels known to occur in response to stress (Goshen and Yirmiya, 2009, Imura *et al.*, 1991). As with IL-6, circulating levels of IL-1β at a time point 24 hours after the stress exposure may not provide a complete picture of events. Therefore, we will now move on to consider lasting

effects of the stress intervention as assessed at target tissue level at a time point 24 hours after a single exposure to restraint.

It has been suggested that plasma concentration of cytokines may not be reliable indicators of cytokine access to the HPA-axis, as it remains unknown whether these concentrations are sufficient to stimulate the HPA-axis under conditions of stress (Turnbull and Rivier, 1995). It therefore seems more plausible to rather measure levels of cytokines within the appropriate tissues.

At tissue level, we assessed the pituitary gland which can be regarded as the stress-related effector region in the brain, integrating all stress afferents innervating the PVN. We also considered the adrenal gland as a representative peripheral target tissue, as well as the source of GCs and cytokines. Prior to processing, masses of the various glands were determined, with the assumption that an increased mass may give a rough indication of increased activity of the particular gland, due to hyperplasia or hypertrophy of activated effector cells (Marti and Armario, 1998, Stokes, 1995).

Pituitary mass did not change in response to stress alone. However, given the fact that this measurement followed a single stress exposure, where an unhindered stress response was possible, i.e. ending with normal negative feedback provided by GCs, one would not expect a lasting change in cell activation. However, when blocking IL-6, a longer lasting response was indeed noticed, with pituitary gland mass increasing (albeit only significant at time point 2), independently of stress. This suggests that when normal homeostasis is threatened, or acutely altered by exogenous factors, immediate adaption or at least compensation may occur.

GR levels in the anterior pituitary did not change in response to stress or blocking IL-6. Although GR levels are known to increase in response to acute stress (Al-Mohaisen *et al.*, 2000, Sun *et al.*, 2002), it is possible that our stress intervention, which was of relatively mild intensity and also intermittent, was not potent to the extend of promoting adaptation at this central level. The fact that GR also did not respond to blocking of IL-6, even in the presence of stress, may suggest that the stressor was so mild that IL-6 levels were never elevated high enough to facilitate or generate a strong pro-inflammatory stimulus for GC release, and thus no adaptation in pituitary GR levels was required after only one exposure. A second possible explanation for this result is that the anti-IL-6 Ab that we administered, was unable to reach the anterior pituitary. However, as the posterior pituitary gland is a circumventricular organ, the Ab did not have to cross the BBB to gain access to the cells (Anisman, 2008), so that it is highly unlikely that the Ab did not reach the tissue. Also, we have demonstrated the presence of the Ab at tissue level in both the pituitary and adrenals. It is therefore more likely that resistance to adaptation exists centrally, especially in response to a stressor of relatively short duration. The same argument would explain the observation that IL-1β, IL-1βR, IL-6, IL-6R and $GABA_AR\alpha1$ showed no response to stress alone.

Both IL-1β and IL-1βR expression increased in the CAb group in the pituitary (as was the case with $GABA_AR\alpha1$), again suggesting a role for IL-6 under control conditions, which is attenuated in the presence of stress. We know from the literature that central regulation is often different to that in the periphery (Bornstein and Chrousos, 1998, Licinio and Wong, 1999, Rivier and Rivest, 1991, Wotjak *et al.*, 1998). Therefore, it is possible that, unlike in the periphery where absence of IL-6 resulted in a decrease in basal IL-1β levels, local IL-1β secretion in the pituitary may

be increased when blocking IL-6, as suggested by our current data (CP vs. CAb, Fig.14). This could be the local effect on diminished IL-1β (and IL-6) negative feedback on cells producing IL-1β in the pituitary, since less IL-1β reached the pituitary *via* the circulation. This would also explain the up-regulation of local IL-1β receptors. The fact that this response was not evident any more in a stressed condition, suggest that the slightly increased corticosterone levels (SAb) from baseline were enough to override this effect, possibly due to the inhibitory effect of GCs on IL-1β.

IL-6 has been reported to stimulate GABA release from the posterior pituitary in rats *in vivo via* stimulation of prostaglandins under stressed conditions, but not in absence of stress (De Laurentiis *et al.*, 2000). This seems to suggest that IL-6 does not influence basal GABA homeostasis. However, our data illustrated an up-regulation of $GABA_AR\alpha1$ after blocking IL-6 in the absence of a stressor. Given the earlier report that IL-6 stimulates rather than suppresses GABA release (albeit in a model of stress), it is unlikely that IL-6 would down-regulate $GABA_AR\alpha1$. In our opinion, our result reflects an indirect effect of blocking IL-6 on GABA regulation *via* the increase in IL-1β and its receptor. Indeed, IL-1β has been shown to enhance the effects of GABA in terms of chloride uptake *in vitro* as well as $GABA_A$ receptor function, possibly *via* the IL-1β receptor (as IL-1β receptor antagonist decreased the effect of IL-1β) (Miller *et al.*, 1991). Also, these effects were reported to occur in favour of IL-6- (and TNF-α-) induced effects on GABA regulation, which would support our interpretation. Taken together, it is clear that more extensive investigations considering the role of GABA and its receptors under conditions of psychological stress are required, especially if one considers the lack of literature on

this topic up to date. Cross-talk between $GABA_AR$ and IL-6 also further remains to be elucidated.

Considering the results obtained in analysis of the adrenal tissue, our results indicate quite different responses peripherally when compared centrally. In terms of adrenal gland mass, blocking IL-6 resulted in decreased glandular mass at time point 1, independent of stress. Our initial interpretation was that the decreased pro-inflammatory signal by IL-6 for GC release in CAb and SAb led to decreased anti-inflammatory activity (GC production) of the adrenal gland. However, when considering the immunohistochemistry data, which indicate roles of both stress and IL-6, it is clear that the answer is not quite as basic.

We gathered from the literature that GR is down-regulated in response to chronic stress (Checkly, 1996) but up-regulated after acute stress. Our result with increased GR in all zones of the adrenal in SP, is in keeping with this literature. Furthermore, our results indicate an effect of Ab on GR independent of stress, with GR increasing when IL-6 is blocked. *In vitro* studies have indicated that IL-6 stimulates GC release from the adrenal glands *via* different mechanisms (Path *et al.*, 2000). Therefore it is possible that the up-regulation in GR levels seen in CAb and SAb is the effect of a compensatory mechanism to increase bioactivity of GCs in conditions where its normal stimulation by IL-6 is compromised. This interpretation is further supported by the fact that this up-regulation is most prominent in the zones responsible for GC production (*zona fasciculate* and *zona reticularis*). However, when considering our cytokine data, a different and novel conclusion can be drawn.

When cytokine protein and receptor expression in the adrenal is considered, no major effect of stress was evident. The effect of blocking IL-6 was similar to GR data

for cytokines assessed. The fact that expression of IL-6, IL-6R, IL-1β and IL-1βR consistently increased in the Ab groups, suggests that lack of local negative cytokine feedback occurred in response to very low plasma IL-6 levels, resulting in increased cytokine and receptor expression. This local IL-6 feedback was previously illustrated in relation to white blood cells in an *in vitro* model (Smith *et al.*, 2007). However, the current study is the first to demonstrate that this feedback loop is also evident at the level of tissues, and the first to demonstrate this using an *in vivo* model. Furthermore, the fact that the zone-specific increases in cytokine and receptor expression were evident in both CAb and SAb groups, and the fact that their expression was not up-regulated during stress alone, suggest that the effect of (at least IL-6) local direct feedback plays a more important role than GCs in the down-regulation of inflammatory cytokine release. This finding has vast implications for our understanding of GC regulation of cytokine-dependent processes.

All receptor levels measured mirrored their protein levels in the tissue in the adrenal gland. The zones that were of interest because of the high amount of fluorescence, particularly the *fasciculata*, were the areas that have been shown to produce corticosterone (Young and Heath, 2004). This notion supports the premise of IL-6 and corticosterone to form a feedback loop as far as their site of production is concerned.

In humans, both IL-6 and IL-6R have revealed to be co-expressed at similar sites in the adrenal gland (Path *et al.*, 2000) which we have confirmed in our study on rats. The up-regulation of IL-6R in the *fasciculata* and *reticularis* when no Ab but stress is present, in addition to the increase in IL-6R expression in these zones in the Ab groups may indicate the requirement for sensitivity to IL-6 action under conditions of stress and lack of IL-6.

Below is an adapted version of the diagram in chapter 1, putting our findings in context with the literature. The relevant tissues where IL-6 exerts its effects are indicated on the diagram by means of green lightning bolts.

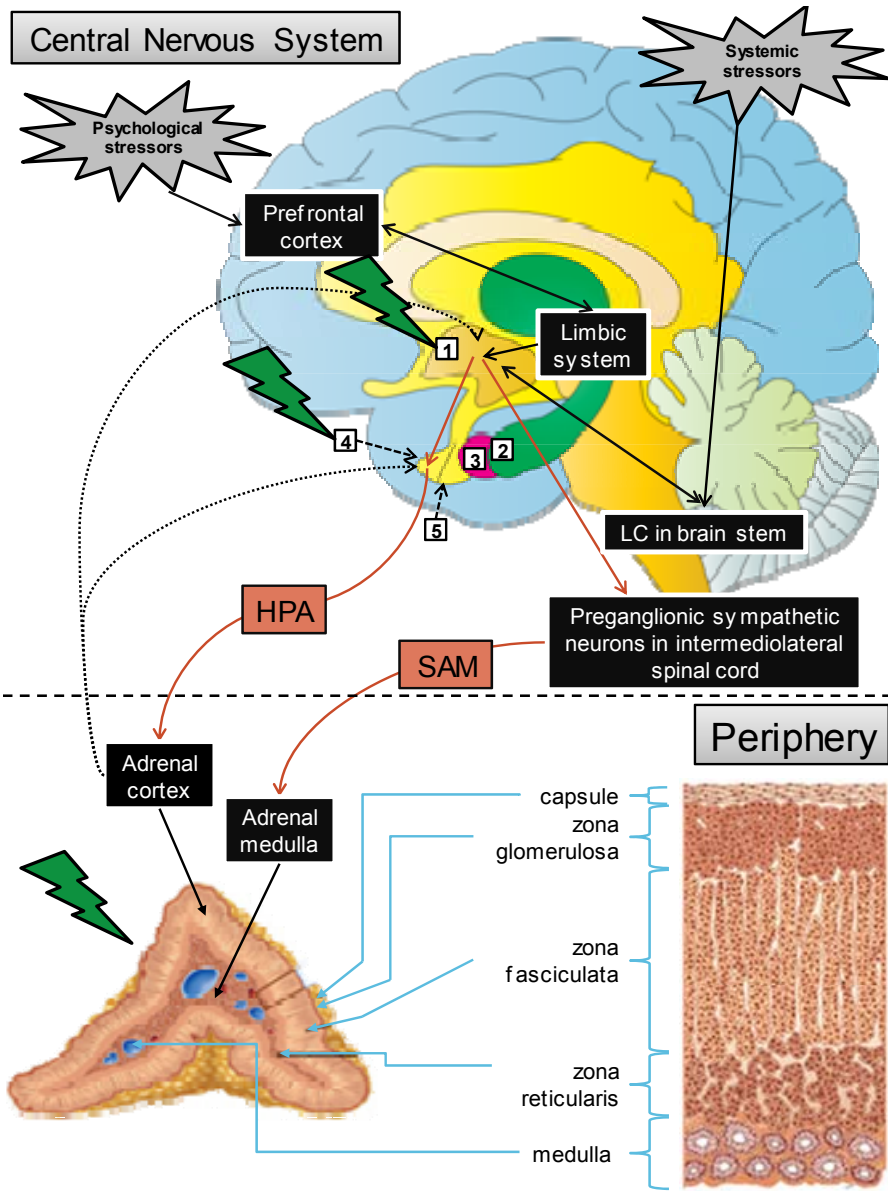

Figure 16: The main brain and adrenal areas involved in the stress response (negative feedback pathways indicated with dashed arrows). Stress stimuli are relayed to the CNS *via* the HPA-axis and the LC system regulating the SAM system. Numbers correspond to brain areas: 1) hypothalamus; 2) hippocampus; 3) amygdala; 4) anterior pituitary; 5) posterior pituitary. The diagram was adapted from the various sources. Abbreviations: LC, Locus coeruleus/norepinephrine system; HPA, hypothalamo-pituitary-adrenal axis; SAM, sympathetic adreno-medullary axis. Green lightning bolts indicate the tissues where IL-6 exerts its effects.

Limitations to the study

As mentioned earlier, the local inflammatory response to injection of either placebo or Ab was found to be a confounder, as reflected by the TNF-α results. This effect can be avoided by habituating the immune system to injection with daily placebo injections of all rats about one week before the onset of the intervention. Alternatively, it may be more ideal to administer the Ab *via* peristaltic pumps implanted subcutaneously, as used in other studies related to cytokine function.

Despite this, we believe that blocking IL-6 acutely is more physiologically representative than the use of an IL-6 knock-out model to investigate IL-6's role in the stress response, due to compensatory alterations in cytokine profiles in knock-outs.

Furthermore, the fact that we did not assess lasting effects at time points 2 and 3 is a limiting factor and needs to be tended to in future studies in order to obtain a complete picture of the responses during repeated stress.

Finally, with regard to tissue analysis, fluorescence decay or variation over time was not corrected for. Ideally, each staining batch should contain a control slide with which a relative correction factor for each day can be calculated from.

Conclusion and directions for future studies

We accepted our hypothesis on the grounds that the HPA response to mild psychological stress did indeed depend on IL-6 and that IL-6 dependent effects were evident 24 hours post intervention, both centrally and peripherally.

Our model of psychological stress has been confirmed to be mild as no lasting adaptation to stress were evident at tissue level. This model then proves to be useful in the investigation of scenarios in the general human population in which the degree of psychological stress is generally mild. In this way our model of stress can be extrapolated to further studies of chronic stress conditions, in order to identify the role of cytokines during chronic, mild stress and the co-existing pathologies such as depression in humans.

Consistent effects of the Ab were apparent in the tissues investigated, even in control conditions, suggesting that IL-6 plays a role in the maintenance of basal homeostasis, including its regulation of psychological stress. The premise that our model of stress was mild, made it possible to deduce the regulation of cytokines during stress without too much interference (effects being masked by GC action).

The contribution of cytokines under basal and stress conditions is important to bear in mind as far as the investigation on psychological stress in humans is concerned, especially since the majority of related studies focus on the regulation of GCs under conditions of stress and to a lesser extend (or none at all) to the role of IL-6 in homeostasis. In practice, stress-related pathologies are normally treated with the aim of limiting cytokine action, which, according to the results of the present study, might interfere with normal cytokine homeostasis. Future studies should therefore

elaborate on what is considered as an ‘optimum’ IL-6 level of expression and release, in order to maintain a balanced cytokine and GC profile.

We found differential regulation in terms of cytokines and GCs when comparing peripheral *versus* central effects of stress and Ab, as well as the levels of cytokines in the blood compartment, compared to within tissues. Future studies should therefore present results in terms of this discrepancy, in order to give a true reflection of the regulation of these mediators.

Lastly, due to financial and time constraints, it was not possible to incorporate an additional behavioural component in the study. However, the outcomes of future studies focusing on behaviour in response to stress (as a psychological measure of stress) can contribute to the integrative approach taken by the current study.

References

1. Abraham E. Effects of stress on cytokine production. *Methods Achiev Exp Pathol* 14: 45-62, 1991.
2. Aguilera G. Corticotropin Releasing Hormone, receptor regulation and the stress response. *TEM* 9: 329-336, 1998.
3. Aguilera G, Kiss A, Lu A, and Camacho C. Regulation of adrenal steroidogenesis during chronic stress. *Endocrine Res* 22: 433-443, 1996.
4. Al-Mohaisen M, Cardounel A, and Kalimi M. Repeated immobilization stress increases total cytosolic glucocorticoid receptor in rat liver. *Steroids* 65: 8-15, 2000.
5. Alexandrova M and Farkas P. Stress-induced changes of glucocorticoid receptor in rat liver. *J Steroid Biochem Mol Biol* 42: 493-498, 1992.
6. Anderson R and Mitchell R. Distribution of GABA binding site subtypes in rat pituitary gland. *Brain Res* 365: 78-84, 1986.
7. Ando T, Rivier J, Yanaihara H, and Arimura A. Peripheral corticotropin-releasing factor mediates the elevation of plasma IL-6 by immobilization stress in rats. *Am J Physiol Regulatory Integrative Comp Physiol* 275: 1461-1467, 1998.
8. Anisman H. Cascading effects of stressors and inflammatory immune system activation: implications for major depressive disorder. . *Rev Psychiatr Neurosci* 34: 4-20, 2008.
9. Axelrod J and Reisine T. Stress hormones: their interaction and regulation. *Science* 224: 452-459, 1984.
10. Banks W, Kastin A, and RD B. Passage of cytokines across the blood-brain barrier. *Neuroimmunomodulation* 2: 241-248, 1995.
11. Banks W, Lynch J, and Price T. Cytokines and the Blood–Brain Barrier. In: *The Neuroimmunological Basis of Behavior and Mental Disorders*, edited by Siegel A and Zalcman SS, 2009, p. 3-17.
12. Bartfai T, Andersson C, Bristulf J, schultzberg M, and senson S. Interleukin-1 in the noradrenergic chromaffin cells in the rat adrenal medulla. *Ann NY Acad Sci*: 207-213, 1990.

13. Bhatnagar S and Dallman M. Neuroanatomical basis for facilitation of hypothalamic-pituitary-adrenal responses to a novel stressor after chronic stress. *Neuroscience* 84: 1025-1039, 1998.
14. Biggio G, Concas A, Corda M, Giorgi O, Sanna E, and Serra M. GABAergic and dopaminergic transmission in the rat cerebral cortex: effect of stress, anxiolytic and anxiogenic drugs. *Pharmacol Ther* 48: 121-124, 1990.
15. Biggio G, Corda M, Concas A, Demontis G, Rossetti Z, and Gessa G. Rapid changes in GABA binding induced by stress in different areas of the rat brain. *Brain Research*: 363-369, 1981.
16. Bornstein S and Chrousos G. Adrenocorticotropin (ACTH)-and non-ACTH-mediated regulation of the adrenal cortex: Neural and immune inputs. . *The Journal of Clinical Endocrinology & Metabolism* 84: 1729-1736, 1998.
17. Boue-Grabot E, Taupignon A, Tramu G, and Garret M. Molecular and electrophysiological evidence for a GABAC receptor in thyrotropin-secreting cells. *Endocrinology* 141: 1627-1632, 2000.
18. Briski K and Gillen E. Differential distribution of Fos expression within the male rat preoptic area and hypothalamus in response to physical vs. psychological stress. *Brain Research Bulletin,* 55: 401-408, 2001.
19. Buam A and Posluszny D. Health psychology: mapping behavioural contributions to health and illness. *Ann Rev Psychol* 50: 137-163, 1999a.
20. Buam A and Posluszny D. Health psychology: mapping behavioural contributions to health and illness. . *Ann Rev Psychol* 50: 137-163, 1999b.
21. Buckingham J, Loxley H, Christian C, and Philip J. Activation of the HPA Axis by Immune Insults: Roles and Interactions of Cytokines, Eicosanoids, and glucocorticoids. *Pharmacol Biochem Behav* 54: 285-298, 1996.
22. Buckingham J, Smith T, and Loxley H. The control of ACTH secretion. *In: James, VHT, ed The adrenal gland, 2nd ed New York: Raven press*: 131-158, 1992.
23. Cacioppo J, Malarkey W, Kiecolt-Glaser J, and al. e. Heterogenity in neuroendocrine and immune responses to brief psychological stressors as a function of autonomic cardiac activation. *Psychosomat Med* 57: 154-164, 1995.
24. Castell J, Geiger T, Gross V, Andus T, Walter E, Hirano T, Kishimoto T, and Henrich P. Plasma clearance, organ distribution, and target cells of

interleukin-6/hepatocyte-stimulating factor in the rat. *Eu J Biochem* 177: 357-361, 1988.

25. Checkly S. The neuroendocrinology of depression and chronic stress. *British Medical Bulletin* 52: 597-617, 1996.
26. Chikanza I and Grossman A. Neuroendocrine immune responses to inflammation: the concept of the neuroendocrine immune loop. *Bailliere's Clinical Rheumatology* 10: 199-225, 1996.
27. Childs G. Structure-function correlates in the corticotropes of the anterior pituitary. *Frontiers in Neuroendocrinology* 13: 271-317, 1992.
28. Chung K, Martinez M, and Herbert J. c-fos expression, behavioural, endocrine and autonomic responses to acute social stress in male rats after chronic restraint: modulation by serotonin. *Neuroscience* 95: 453-463, 2000.
29. Coelho M, Luheshi S, Hopkins S, Pela I, and Rothwell N. Multiple mechanisms mediate antipyretic action of glucocorticoids. *Am J Physiol* 269: 527-535, 1995.
30. Cole R and Sawchenko P. Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. *J Neurosci* 22: 959-969, 2002.
31. Collins G. GABA-2-oxygluterate transaminase, glutamate decarboxylase and the half-life of GABA in different areas of the rat brain. *Biochem Pharmacol* 21: 2849-2858, 1972.
32. Cullinan W and Wolfe T. Chronic stress regulates levels of mRNA transcripts encoding -subunits of the GABAA receptor in the rat stress axis. *Brain Res* 887: 118-124, 2000.
33. Day T, Buller K, Xu Y, Dayas C, and Crane J. Separation of neural pathways mediating HPA axis responses to emotional and physical stressors. *Exerpta Med Congr Series* 1185: 213-221, 1999.
34. De Laurentiis A, Pisera D, Lasaga M, Dias M, Theas S, Duvilanski B, and Seilicovich A. Effect of interleukin-6 and tumor necrosis factor-alpha on GABA release from the mediaobasal hypothalamus and posterior pituitary. *Neuroimmunomodulation* 7: 77-83, 2000.
35. De Souza L and Franci C. GABAergic mediation of stress-induced secretion of corticosterone and oxitocin, but not prolactin, by the hypothalamic paraventricular nucleus. *Life Sciences* 83: 686-692, 2008.

36. Deak T, Bordner K, McElderry N, Barnum C, Blandino Jr P, Deak M, and Tammariello S. Stress-induced increases in hypothalamic IL-1: a systematic analysis of multiple stressor paradigms. *Brain Research Bulletin* 64: 541-556, 2004.
37. DeRijk R, Michelson D, Karp B, Petrides J, Galliven E, Deuster P, Paciotti G, Gold P, and Sternberg E. Exercise and circadian rhythm-induced variations in plasma cortisol differentially regulate interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) production in humans:high sensitivity if TNF-α and resistance of IL-6. *J Clin Endocrinol Metab* 82: 2182-2191, 1997.
38. Dinaerello C. The proinflammatory cytokines interleukin-1 and tumor necrosis factor and treatment of the septic shock syndrome. *J Infect Dis* 163: 1177-1184, 1991.
39. Duvilanski B, Perez R, Seilicovich A, Lasaga M, Diaz M, and Debeljuk L. Intracellular distribution of GABA in the rat anterior pituitary. An electron microscopic autoradiographic study. *Tissue Cell* 32: 284-292, 2000.
40. Ebisui O, Fukata J, Murakami N, Kobayashi H, Segawa H, Muro S, HAnaoka Y, Naito Y, Masui Y, Ohmoto Y, Imura H, and Nakao K. Effect of IL-1 receptor antagonist and antiserum to TNF-a on LPS-induced plasma ACTH and corticosterone rise in rats. *Am J Physiol* 266: 986-992, 1994.
41. Elenkov I and Chrousos G. Stress, cytokine patterns and susceptibility to disease. *Bailliere's Clinical Endocrinology and Metabolism* 13: 583-595, 1999.
42. Emmert M and Herman J. Differential forebrain c-fos mRNA induction by ether inhalation and novelty: evidence for distinctive stress pathways. *Brain Res* 845: 60-67, 1999.
43. Eskay R, Grino M, and Chen H. Interleukens, signal transduction, and the immune system-mediated stress response. *Circulating Regulatory Factors and Neuroendocrine Function* 21: 331-343, 1990.
44. Feldman S, Conforti N, and Weidenfield J. Limbic pathways and hypothalamic neurotransmitters mediating adrenocortical responses to neural stimuli *Neuroscience and Biobehavioral reviews* 19: 235-239, 1995.
45. Fernandes G, Perks P, Cox N, Lightman S, Ingram C, and Shanks N. Habituation and Cross-Sensitization of Stress-Induced Hypothalamic-Pituitary-Adrenal Activity: Effect of Lesions in the Paraventricular Nucleus of

the Thalamus or Bed Nuclei of the Stria Terminalis. *Journal of Neuroendocrinology,* 14: 593-602, 2002.

46. Fink G. Encyclopedia of stress. San Diego: Academic Press. 2000.
47. Fleshner M, Goehler L, Herman J, Relton J, Maier S, and Watkins L. Interleukin-1 beta induced corticosterone elevation and hypothalamic NE depletion is vagally mediated. *Brain Res Bull* 44: 701-706, 1995.
48. Furay A, Bruestle A, and Herman J. The Role of the Forebrain Glucocorticoid Receptor in Acute and Chronic Stress. *Endocrinology* 149: 5482-5490, 2008.
49. Gadient R, Lachmund A, Unsicker K, and U O. Expression of interleukin-6 (IL-6) and IL-6 receptor mRNAs in rat adrenal medulla. . *Neurosci Lett* 194: 17-20, 1995.
50. Garcia A, Martı´ O, Valles A, Dal-Zotto S, and Armario A. Recovery of the hypothalamic-pituitary-adrenal response to stress. Effect of stress intensity, stress duration and previous stress exposure. *Neuroendocrinology* 72: 114-125, 2000.
51. Gaykema R, Dijkstra I, and Tilders F. Subdiaphragmatic vagotomy suppresses endotoxin-induced activation of hypothalamic corticotropin-releasing hormone neurons and ACTH secretion. *Endocrinology* 136: 4717-4720, 1995.
52. González-Hernández J, Bornstein S, Ehrhart-Bornstein M, Späth-Schwalbe E, Jirikowski G, and Scherbaum W. Interleukin-6 messenger ribonucleic acid expression in human adrenal gland in vivo. New clue to a paracrine or autocrine regulation of adrenal function. . *J Clin Endocrinol Metab* 79: 1492-1497, 1994.
53. Goshen I and Yirmiya R. Interleukin-1 (IL-1): A central regulator of stress responses. *Frontiers in Neuroendocrinology* 30: 30-45, 2009.
54. Haddad J, Saade N, and B S-G. Cytokines and neuro–immune–endocrine interactions: a role for the hypothalamic–pituitary–adrenal revolving axis. *Journal of Neuroimmunology* 133: 1-19, 2002.
55. Hauger R, Lorang M, Irwin M, and Aguilera G. CRF receptor regulation and sensitization of ACTH responses to acute ether stress during chronic intermittent immobilization stress. *Brain Res* 532: 34-40, 1990.
56. Herman J and Cullinan W. Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci* 20: 78-84, 1997.

57. Herman J, Dolgas C, and Carlson S. Ventral subiculum regulates hypothalamo-pituitary-adrenocortical and behavioural responses to cognitive stressors. *Neuroscience* 86: 449-459, 1998.
58. Herman J, Figueiredo H, Mueller N, Ulrich-Lai Y, Ostrander M, Choi D, and Cullinan W. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo–pituitary–adrenocortical responsiveness. . *Frontiers in Neuroendocrinology* 24: 151–180, 2003.
59. Herman J, Meuller N, and Figueiredo H. Role of GABA and glutamate circuitry in hypothalamo-pituitary-adrenocortical stress integration. *Ann NY Acad Sci* 1018: 35-45, 2004.
60. Hinz B and Hirschelmann R. Rapid non-genomic feedback effects of glucocorticoids on CRF-induced ACTH secretion in rats. *Pharm Res* 17: 1273-1277, 2000.
61. Hu Y, Cardounel A, Gursoy E, Anderson P, and Kalimi M. Anti-stress effects of dehydroepiandrosterone: protection of rats against repeated immobilization stress-induced weight loss, glucocorticoid receptor production, and lipid peroxidation. *Biochem Pharmacol* 59: 753-762, 2000.
62. Imura H, Fukata J, and Mori T. Cytokines and endocrine function: an interaction between the immune and neuroendocrine systems. *Clinical Endocrinology* 35: 107-115, 1991.
63. Jacobson L and Sapolsky R. The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocr Rev* 12: 118-134, 1991.
64. Jain S, Mills P, Von Kanel R, Hong S, and Dimsdale J. Effect of perceived stress and uplifts on inflmmation and coagubility. *Psychophysiology* 44: 154-160, 2007.
65. John C and Buckingham J. Cytokines: regulation of the hypothalamo-pituitary-adrenocortical axis. *Current Opinion in Pharmacology* 3: 78-84, 2003.
66. Joseph J, Grun J, and Lublin FK, RL. Interleukin-6 induction in vitro in mouse brain endothelial cells and astrocytes by exposure to mouse hepatitis virus. *J Neuroimmunol* 42: 47-52, 1993.
67. Judd A and Macleod R. Adrenocorticotropin increases interleukin-6 release from rat adrenal zona glomerulosa cells. *Endocrinology* 190: 1245-1254, 1992.

68. Judd A and Macleod R. Angiotensin II increases interleukin-6 release from rat adrenal zona glomerulosa cells. . *Prog Neuroendocrin Immunol* 4: 240-247, 1991.
69. Judd A, Spangelo B, and Macleod R. Rat adrenal zona glomerulosa cells produce interleukin 6. . *Prog Neuroendocrin Immunol* 3: 282-292, 1990.
70. Koenig J, Snow K, Clark B, Toni R, Cannon J, Show A, Dinarello C, Reichlin S, Lee S, and Lechan R. Intrinsic pituitary interluekin-1β is induced by bacterial lipopolysaccharide. *Endocrinology* 126: 3053-3058, 1990.
71. Kovacs K, Miklos I, and Bali B. GABAergic mechanisms constraining the activity of the hypothalamo-pituitary-adrenocortical axis. *Ann NYAcadSci* 1018: 466-476, 2004.
72. Kronfol Z and Remick D. Cytokines and the brain: Implications for clinical psychiatry. *Am J Psychiatry* 157: 683-694, 2000.
73. Laye S, Bluthe R, Kent S, Combe C, Medina C, Paarnet P, Kelly K, and Dantzer R. Subdiaphragmatic vagotomy blocks induction of IL-1 beta mRNA in mice brain in response to peripheral LPS. *Am J Physiol* 268: 1327-1331, 1995.
74. Laye S, Parnet P, Goujon E, and Dantzer R. Peripheral administration of lipopolysaccharide induces the expression of cytokine transcripts in the brain and pituitary of mice. *Brain Research Molecular Brain Research* 27, 1994.
75. Lee S, Tsou A, Chan H, Thomas J, Petrie K, Eugui E, and Allison A. Glucocorticoids selectively inhibit the transcription of the Interleukin-1 gene and decrease the stability of Interleukin 1 mRNA. *Proc Natl Acad Sci USA* 85: 1204-1208, 1988.
76. LeMay LG, Vander A, and Kluger M. The effects of psychological stress on plasma interleukin-6 activity in rats. *Physiol Behav* 47: 957-961, 1990.
77. Liao J, Keiser J, Scales W, Kunkel S, and Kluger M. Role of corticosterone in TNF and IL-6 productio in isolated perfused rat liver. *Regulatory Integrative Comp Physiol* 37: 699-706, 1995.
78. Licinio J and Frost P. The neuroimmune-endocrine axis: pathophysiological implications for the central nervous system cytokines and hypothalamus-pituitary-adrenal hormone dynamics. *Braz J Med Biol Res* 33: 1141-1148, 2000.

79. Licinio J and Wong M-L. The role of inflammatory mediators in the biology of major depression: central nervous system cytokines modulate the biological substrate of depressive symptoms, regulate stress-responsive systems, and contribute to neurotoxicity and neuroprotection. *Molecular Psychiatry* 4: 317-327, 1999.

80. Lovallo W and Thomas T. Handbook of psychophysiology. edited by Cacioppo J, Tassinary L and Berntson G: Cambridge University press, Cambridge, UK, 2000.

81. Lozovaya N and Miller A. Bidirectional communication between immune and stress (Limbic-Hypothalamic-Pituitary-Adrenal) systems. . *ChemBioChem* 4: 466-484, 2003.

82. Ma X, Lightman S, and G A. Vasopressin and corticotropinreleasing hormone gene responses to novel stress in rats adapted to repeated restraint. *Endocrinology* 140: 3623-3632, 1998.

83. Maier S and Watkins L. Cytokines for psychologists: Implications of bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. *Psychol Rev* 105: 83-107, 1998.

84. Makino S, Schulkin J, Smith M, Pacak K, Palkovits M, and Gold P. Regulation of corticotropin-releasing hormone receptor messenger ribonucleic acid in the rat brain and pituitary by glucocorticoids and stress. *Endocrinology* 136: 4517-4525, 1995.

85. Marti O and Armario A. Anterior pituitay response to stress: time-related changes and adaptation. *Int J Devl Neuroscience* 16: 241-260, 1998.

86. Martı´ O, Harbuz M, Andre´s R, Lightman S, and Armario A. Activation of the hypothalamic-pituitary-adrenal axis in adrenalectomised rats: potentiation by chronic stress. *Brain Res* 824: 1-7, 1999.

87. Mastorakos G, Chrousos G, and Weber J. Recombinant interleukin-6 activates the hypothalamic-pituitary-adrenal axis in humans. *Clin Endocrinol Metabolism* 77: 1690-1694, 1993.

88. Mayerhofer A, Hoehne-Zell B, and Gamel-Didelon K. Gamma-aminobutyric acid (GABA): a para- and/or autocrine hormone in the pituitary. *FASEB J* 15: 1089-1091, 2001.

89. Melia K, Ryabinin A, Schroeder R, Bloom F, and Wilson M. Induction and habituation of immediate early gene expression in rat brain by acute and repeated restraint stress. *J Neurosci* 14: 5929-5938, 1994.
90. Mikkelsen J, Bundzikova J, Larsen M, Hansen H, and Kiss A. GABA regulates the rat Hypothalamic-Pituitary-Adrenocortical Axis via different GABA-A receptor α-subtypes. *Ann NY Acad Sci* 1148: 384-392, 2008.
91. Miller D and O'Callaghan J. Depression, cytokines, and glial function. *Metabolism Clinical and Experimental* 54: 33-38, 2005.
92. Miller D and O'Callaghan J. Neuroendocrine aspects of the response to stress. . *Metabolism* 51: 5-10, 2002.
93. Miller L, Galpern W, Dunlap C, Dinarello C, and Turner T. Interleukin-1 augments GABA-A receptor function in brain. *Mol Pharmacol* 39: 105-108, 1991.
94. Minami M, Kuraishi Y, Yamaguchi T, Nakai S, Hirai Y, and Satoh M. Immobilization stress induces interleukin-1b mRNA in the rat hypothalamus. *Neurosci Lett* 123: 254-256, 1991.
95. Munck A and Naray-Fejes-Toth A. Glucocorticoids and stress: permissive and suppressive actions. *Ann NYAcadSci* 746: 115-133, 1994.
96. Naitoh Y, Fukata T, Tominaga Y, Nakai S, Tamai S, Mori K, and Imura H. Interleukin-6 stimulates the secretion of adrenocorticotropic hormone in conscious, freely-moving rats. *Biochem Biophys Res Commun* 155: 1459-1463, 1988.
97. Nguyen K, Deak T, Will M, Hansen M, Hunsaker B, Fleshner M, Watkins L, and Maier S. Timecourse and corticosterone sensitivity of the brain, pituitary, and serum interleukin-1 protein response to acute stress. *Brain Res* 859: 193-201, 2000.
98. Nishimura K, Makino S, Tanaka Y, Kaneda T, and Hashimoto T. Altered expression of p53 mRNA in the brain and pituitary during repeated immobilization stress: negative correlation with glucocorticoid receptor mRNA levels. *J Neuroendocrinol* 16: 84-91, 2004.
99. Nukina H, SUdo N, Aiba Y, Oyama N, Koga Y, and Kubo C. Restraint stress elevates the plasma interleukin-6 levels in germ-free mice. *J Neuroimmunol* 115: 46-52, 2001.

100. Nukina H, SUdo N, Komaki G, Yu X, Mine K, and Kubo C. The restraint stress-induced elevation in plasma interleukin-6 negatively regulates the plasma TNF-a level. *Neuroimmunomodulation* 5: 323-327, 1998a.
101. Nukina H, Sudo N, Komaki G, Yu X, Mine K, and Kubo C. The restraint stress-induced elevation in plasma interleukin-6 negatively regulates the plasma TNF-alpha level. *Neuroimmunomodulation* 5: 323-327, 1998b.
102. O'Conner K, Johnson J, Hammack S, Brooks L, Spencer R, Watkins L, and Maier S. Inescapable shock induces resistance to the effects of dexamethasone. *Psychoneuendocrinology* 28: 481-500, 2003.
103. O'Brien S, Scott L, and TG D. Cytokines: abnormalities in major depression and implications for pharmacological treatment. . *Hum Psychopharmacol Clin Exp* 19: 397-403, 2004.
104. O'Connor K, Johnson J, Hansen M, Frank J, Maksimova E, Watkins L, and Maier S. Peripheral and central proinflammatory cytokine response to a severe acute stressor. *Brain Research* 991: 123-132, 2003.
105. O'Connor T, O'Halloran D, and Shanahan F. The stress response and the hypothalamic-pituitary-adrenal axis: from molecule to melancholia. *Q J Med* 93: 323-333, 2000.
106. Ohmichi M, Hirota K, Koike K, Kurachi H, Ohtsuka S, Matsuzani N, Yamaguchi M, Miyake A, and Tanizawa O. Binding sites for interleukin-6 in the anterior pituitary gland. *Neuroendocrinology* 55: 199-203, 1992.
107. Okret S, Dong Y, Bronnegard M, and Gustaffson J. Regulation of glucocorticoid expression. *Biochimie* 73: 51-59, 1991.
108. Otero Losada M. Changes in central GABAergic function following acute and repeated stress. *Br J Pharmacol* 93: 483-490, 1988.
109. Path G, Bornstein S, Ehrhart-Bornstein M, and Scherbaum W. Interleukin-6 and the Interleukin-6 Receptor in the human adrenal gland: expression and effects on steroidgenesis. *Jounal of Clinical Endocrinology and Metabolism* 82: 2343-2349, 1997.
110. Path G, Scherbaum W, and SR B. The role of interleukin-6 in the human adrenal gland. *European Journal of Clinical Investigation* 30: 91-95, 2000.
111. Petrides J, Gold P, Mueller G, Singh A, Stratakis C, Chrousos G, and Deuster P. Marked differences in functioning of the hypothalamicpituitary-adrenal axis between groups of men. *J Appl Physiol* 82: 1979-1988, 1997.

112. Pitman D, Ottenweller J, and BH N. Effect of stressor intensity on habituation and sensitization of glucocorticoid responses in rats. *Behav Neurosci* 104: 28-36, 1990.
113. Pitman D, Ottenweller J, and Natelson B. Plasma corticosterone levels during repeated presentation of two intensities of restraint stress: chronic stress and habituation. *Physiol Behav* 43: 47-55, 1987.
114. Plata-Salama C, Ilyin S, Turrin N, Gayle D, Flynn M, Bedard T, Merali Z, and Anisman H. Neither acute nor chronic exposure to a naturalistic (predator) stressor influences the interleukin-1β system, tumor necrosis factor-α, transforming growth factor-β1, and neuropeptide mRNAs in specific brain regions. *Brain Research Bulletin* 51: 187-193, 2000.
115. Rabadan-Diehl C, Kiss A, Camacho C, and Aguilera G. Regulation of messenger ribonucleic acid for corticotropin releasing hormone receptor in the pituitary during stress. *Endocrinology* 137: 3808-3814, 1996.
116. Rabpoport S. Blood-brain barrier in Physiology and Medicine. *New York: Raven*, 1976.
117. Rapoport S. Blood Brain Barrier in Physiology and Medicine. *Raven Press, New York*, 1976.
118. Rasband W. ImageJ, U. S. (1.41o ed.). Bethesda, Maryland, USA: National Institutes of Health, 1997-2009.
119. Reichlin S. Neuroendocrine-immune interactions. *New England Journal of Medicine* 329: 1246-1253, 1993.
120. Resnikov L, Reagen L, and Fadel J. Effects of acute and repeated restraint stress on GABA efflux in the rat basolateral and central amygdala. *Brain Res* 1256: 61-68, 2008.
121. Reyes T and Coe C. The proinflammatory network: interactions in the CNS and blood of rhesus monkeys. *Am J Physiol* 274: 139-144, 1998a.
122. Reyes T and Coe C. Resistance of central nervous system interleukin-6 to glucocorticoid inhibition in monkeys. *Am J Physiol* 275: 612-618, 1998b.
123. Ringheim G, Burgher K, and Heroux J. Interleukin-6 mRNA expression by cortical neurons in culture: evidence for neuronal sources of interleukin-6 production in the brain. *J Neuroimmunol* 63: 113-123, 1995.

124. Rivier C and Rivest S. Effect of Stress on the Activity of the Hypothalamic-Pituitary-Gonadal Axis: Peripheral and Central Mechanisms. *Biology od reproduction* 45: 523-532, 1991.
125. Rivier C and Vale W. Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. *Nature* 305: 325-327, 1983.
126. Robles T, Glaser R, and Kiecolt-Glaser J. Out of Balance: A New Look at Chronic Stress, Depression, and Immunity. . *Current directions in Psychological Science* 14: 111-115, 2005.
127. Sapolsky R, Romero L, and Munck A. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory and preperative Actions. *Endocrine Reviews* 21: 55-89, 2000.
128. Schimchowitsch S, Vuillez P, and Tappaz M. Systematic presence of GABA-immunoreactivity in the tuberoinfundibular and tubero-hypophyseal dopaminergic axonal systems: an ultrastructural immunogold study on several mammals. *Exp Brain Res* 83: 575-586, 1991.
129. Schindler R, Mancilla J, Endres S, Ghorbani R, Clark S, and Dinarello C. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumour necrosis factor in human blood mononucleur cells: IL-6 Suppresses IL-1 and TNF. *Blood* 75: 40-47, 1990.
130. Schobitz B, Van Den Dobbelsteen M, Holsboer F, Susanto W, and De Kloet E. Regulation of interleukin-6 gene expression in rat. *Endocrinology* 132: 1569-1576, 1993.
131. Scultzberg M, Tingsborg S, Nobel S, Lundkvist J, Svenson S, Simoncsits A, and Bartfai T. Interleukin-1 receptor antagonist protein and mRNA in the rat adrenal gland. *J Interferon Cytokine Res* 15: 721-729, 1995.
132. Sgoutas-Emch S, Cacioppo J, Uchino B, Malarkey W, Pearl D, Kiecolt-Glaser J, and R G. The effects of an acute psychological stressor on cardiovascular, endocrine, and cellular immune response: a prospective study of individuals high and low in heart rate reactivity. *Psychophysiology* 31: 264-271, 1994.
133. Shaftel S, Griffen W, and O'Banion M. The role of interleukin-1 in neuroinflammation and Alzheimer disease: and evolving perspective. *Journal of Neuroinflammation* 5: 1-12, 2008.

134. Shalaby M, Waage A, Aarden L, and Espevick T. Endotoxin, tumor necrosis factor-α and interleukin-1 induce interleukin-6 production in vivo. *Clin Immunol, Immunopathol* 53: 3131-3133, 1989.
135. Sharp B, Matta S, Peterson P, Newton R, Chao C, and McAllen K. Tumour necrosis factor-α is a potent ACTH secretegogue: comparison to interleukin-1β. *Endocrinology* 124: 3131-3133, 1989.
136. Shepard R and Shek P. Immune responses to inflammation and trauma: a physical training model. *Can J Physiol Pharmacol* 76: 469-472, 1998.
137. Sherwood L. In: *Human Physiology: From cells to systems* (Fifth addition ed.), edited by Julet M: Thomson Brooks/cole, 2004.
138. Shintani F, Nakai T, kanba S, Koichi S, Yagi G, Shiozawa M, Also S, Kato R, and Asai M. Involvement of interleukin-1 in immobilization stress-induced increase in plasma adrenocorticotropic hormone and in release of hypothalamic catecholamines in the rat. *J Neurosci* 15: 1961-1970, 1995.
139. Singh A, Petrides J, Gold P, Chrousos G, and Deuster P. Differential Hypothalamic-Pituitary-Adrenal Axis Reactivity to Psychological and Physical Stress. *The Journal of Clinical Endocrinology & Metabolism* 84: 1944-1948, 1999.
140. Skerritt J, Trisdikoon P, and Johnston G. Increased GABA binding in mouse brain following acute swim stress. *Brain Res* 215: 398-403, 1981.
141. Smith C. *Exercise, stress and immune system functional responses*. Stellenbosch: Stellenbosch University, 2004.
142. Smith C, Wilson N, Louw A, and Myburgh K. Illuminating the interrelated immune and endocrine adaptations after multiple exposures to short immobilization stress by in vivo blocking of IL-6. *Am J Physiol Regulatory Integrative Comp Physiol* 292: 1439-1447, 2007.
143. Smith C, Wilson N, Louw A, and Myburgh K. Illuminating the interrelated immune and endocrine adaptations after multiple exposures to short immobilization stress by in vivo blocking of IL-6. *Am J Physiol Regulatory Integrative Comp Physiol* 292: 1439-1447, 2006.
144. Song D, Suh H, Huh S, Jung J, iIhn B, Choi I, and Kim Y. Central GABA-A and GABA-B receptor modulation of basal and stress-induced plasma interleukin-6 levels in mice. *The Journal of Pharmacology and Experimental Therapeutics* 287: 144-149, 1998.

145. Spangelo B, Horrel S, Goodwin A, and Jarvis W. Somatostasin and gamma-aminobutyric acid inhibit interleukin-1 beta-stimulated release of interleukin-6 from rat C6 glioma cells. *Neuroimmunomodulation* 11: 332-340, 2004.
146. Spath-Schwalbe E, Born J, Schrezenmeier H, Bornstein S, Stromeyer P, Drechler S, and al. e. Interleuken- 6 stimulates the hypothalamus-pituitary-adrenal axis in man. . *J Clin Endocrinol Metab* 79: 1212-1214, 1994.
147. Sternberg E, Crousos G, Wilder R, and PW G. The stress response and the regulation of inflammatory disease. *Ann, Intern Med* 117: 854-866, 1992.
148. Stokes P. The potential role of excessive cortisol induced by HPA hyperfunction in the pathogenesis of depression. *European Neuropsychopharmacology Supplement*: 77-82, 1995.
149. Sun X, Fischer D, Pritts T, Wray C, and Hasselgren P. Expression and binding activity of the glucocortioid receptor are upregulated in septic muscle. *Am J Physiol Regulatory Integrative Comp Physiol* 282: 509-518, 2002.
150. Swanson L. Brain Architecture: Understanding the basic plan. *Oxford Unniversity press*, 2003.
151. Takaki A, Huang Q-H, Somogyvri-Vigh A, and Arimura A. Immobilization stress may increase plasma interleukin-6 via central and peripheral catecholamines. *Neuroimmunomodulation* 1: 335-342, 1994.
152. Tataki A, Huang Q, Somogyvari-Vigh A, and Arimura A. Immobilization stress may increase plasma interleukin-6 via central and peripheral catecholamines. *Neuroimmunomodulation* 1: 335-352, 1994.
153. Taylor S. Health psychology. edited by Hope R: Boston: McGrew-Hill, 2003, p. 178-191.
154. Tosato G and Jones K. Interleukin-1 induces interleukin-6 production in peripheral blood monocytes. *Blood* 75: 1305-1310, 1990.
155. Turnbull A and Rivier C. Regulation of the HPA Axis by cytokines. *Brain Behav Immun* 9: 253-275, 1995.
156. Turnbull A and Rivier C. Regulation of the Hypothalamic-Pituitary-Adrenal Axis by Cytokines: Actions and Mechanisms of Action. *Physiological reviews* 79: 2-43, 1999.
157. Verkuyl J, Hemby S, and Joels M. Chronic stress attenuates GABAergic inhibition and alters gene expression of parvocellular neurons in rat hypothalamus. *Eur J Neurosci* 20: 1665-1673, 2004.

158. Verkuyl J, Karst H, and M J. GABAergic transmission in the rat paraventricular nucleus of the hypothalamus is suppressed by corticosterone and stress. *Eur J Neurosci* 21: 113-121, 2005.
159. Vilcek J. The cytokines: an overview. *The Cytokine Handbook, 4th Edition Editor MT Thompson, Elsevier, Amsterdam*, 2003.
160. Vincent S, Hokfelt T, and Wu J. GABA neuron systems in hypothalamus and the pituitary gland: immunohistochemical demonstration using antibodies against glutamate decarboxylase. *Neuroendocrinology* 34: 117-125, 1982.
161. Waage A, Slupphaug G, and Shalaby R. Glucocorticoids inhibit the production of IL-6 from monocytes, endothelial cells, and fibroblasts. *Eur J Immunol* 20: 2439-2443, 1990.
162. Weigent D and Blalock J. Associations between the neuroendocrine and immune systems. *J Leukoc Biol* 57: 137-150, 1995.
163. Wilson N. *Effects of neutralising interleukin-6 on glucocorticoid-mediated adaptations to stress in rat skeletal muscle and liver*. Stellenbosch: University of Stellenbosch, 2005.
164. Wotjak C, Ganster J, Kohl G, Holsboer F, Landgraf R, and Engelmann M. Dissociated central and peripheral release of vasopressin, but not oxytocin, in response to repeated swim stress: new insights into the secretory capacities of peptidergic neurons. *Neoroscience* 85: 1209-1222, 1998.
165. Yoneda Y, Kanmori K, Ida S, and Kuriyama K. Stress induced alterations in the metabolism of γ-aminobutyric acid in rat brain. *J Neurochem* 40: 350-360, 1983.
166. Young B and Heath J. Wheather's Functional Histology. 319-323, 2004.
167. Zemkova H, Bjelobaba I, Tomic M, Zemkova H, and Stojilkovic S. Molecular, pharmacological and functional properties of GABA-A receptors in anterior pituitary cells. *J Physiol* 586: 3097-3111, 2008.
168. Zhang M, Hashimoto T, and Kuriyama K. Stress induced alterations in the striatal GABA-A receptor complex. *Japan J Pharmacol* 53: 121-124, 1990.
169. Zhou D, Kusnekov A, Shurine M, Depaoli M, and Rabin B. Exposure to physical and psychological stressors elevates plasma interleukin-6: Relationship to the activation of the hypothalamic-pituitary-adrenal axis. *Endocrinology* 133: 2523-2530, 1993.

170. Zhou D, Shanks N, Riechman S, Liang R, Kusnecov A, and Rabin B. Interleukin 6 modulates interleukin-1 and stress-induced activation of the hypothalamic-pituitary- adrenal axis in male rats. *Neuroendocrinology* 63: 227-236, 1996.

171. Zuckerman S, Sellhaas J, and Butler L. Differential regulation of lipopolysacccharide-induced interleukin-1 and tumour necrosis factor synthesis: effects of endogenous and exogenous glucocorticoids and the role of the pituitary -adrenal axis. *Eur J Immunol* 19: 301-305, 1989.

Appendix A: Automatic tissue processing

Reagents

1. Alcohol (70 %, 90 %, 95 %, 100 %)
2. Xylene – Sigma-Aldrich
3. Paraffin wax – Merck, Histosec melting point 56 °C

Method

Processing time:

A) Dehydration

1) 70 % alcohol – 1.5 hr
2) 70 % alcohol – 1.5 hr
3) 90 % alcohol – 1.5 hr
4) 95 % alcohol – 1.5 hr
5) 95 % alcohol – 1.5 hr
6) 100 % alcohol – 1.5 hr
7) 100 % alcohol – 1.5 hr
8) 100 % alcohol – 2.0 hr

B) Clearing

9) Xylene – 1.5 hr
10) Xylene – 2.0 hr

C) Impregnation

11) Paraffin wax – 2.0 hr
12) Paraffin wax – 2.0 hr

Thus Total processing time = 20 hr

Appendix B: H&E staining protocol

Reagents

1. 10 % Acid alcohol
    - 10 ml 1 % HCl dissolved in 1 ℓ 70 % alcohol
2. Alcohol (70 %, 95 %, 100 %)
3. Eosin

Stock solution:

10 g Eosin dissolved in 1 ℓ distilled water

Working solution:

10 ml Eosin stock solution dissolved in 90 ml distilled water.

Prepare fresh daily.

For staining:

Add 2 – 3 drops glacial acetic acid per 100 ml before use.

4. Haematoxylin
    - 5 g Harris haematoxylin
    - 100 g Ammonium Alum
    - 50 ml 100 % alcohol
    - 1 ℓ distilled water
    - 2.5 g Mercuric oxide

To prepare: Dissolve haematoxylin in alcohol.

Add Ammonium Alum to distilled water and heat to boiling point.

Immediately add mercuric Oxide and shake until solution has purple-black colour.

Cool rapidly in fridge.

For staining:

Filter before use.

Add 4 ml glacial acetic acid per 100 ml of haematoxylin.

5. Scott's tap water
    - 3.5 g $NaHCO_3$
    - 20 g $MgSO_4$
    - 10 ml 37 % Formalin
    - 1 ℓ tap water

To prepare:

Dissolve $NaHCO_3$ in tap water first.

Add $MgSO_4$ and formalin.

6. Xylene Method

1. Xylene (10 min)
2. 100 % alcohol (10 dips)
3. 100 % alcohol (10 dips)
4. 95 % alcohol (10 dips)
5. 95 % alcohol (10 dips)
6. 70 % alcohol (10 dips)
7. Rinse in distilled water
8. Haematoxylin (3 min)
9. Rinse in distilled water
10. Rinse in acid alcohol
11. Rinse in distilled water
12. Blue in Scott's tap water
13. Rinse distilled water
14. 2 min in Eosin
15. Rinse in distilled water
16. 70 % alcohol (10 dips)
17. 95 % alcohol (10 dips)

18. 95 % alcohol (10 dips)
19. 100 % alcohol (10 dips)
20. 100 % alcohol (10 dips)
21. Xylene (10 dips)
22. Xylene (10 dips)
23. Mount with coverslip

Appendix C: Conventional deparaffinization and dehydration sequence of paraffin embedded tissue prior to immunohistochemistry.

Reagents

1. Alcohol (50 %, 80 %, 95 %, 100 %)
2. Xylene – Sigma-Aldrich

Method

1. Incubate sections in Xylene: 2 changes, 5 min each
2. 100 % absolute alcohol: 2 changes, 3 min each
3. 95 % alcohol: 2 changes, 3 min each
4. 80 % alcohol: 3 min
5. 50 % alcohol: 3 min
6. Rinse in distilled water: 2 changes, 3 min each

Appendix D: Immunohistochemistry staining procedure (2 markers)

Reagents

1. PBS, pH 7.4, 1 ℓ of 1 M phosphate buffer, 90 g NaCl, 9 ℓ dH_2O
2. Donkey serum – Jackson Immunoresearch Inc.
3. For antibodies used, see Table 1

Method

1. Wash slides in PBS.
2. Dry, encircle samples with a wax pen.
3. Block for 30 min in 5% serum at room temperature (RT). (*Note: use the same serum in which the secondary Ab is raised*)
4. Shake off serum and incubate sections for 4 hr at RT with the 1st primary Ab. (*Note: Do not wash after serum blocking step*)
5. Wash slides with PBS and add the secondary Ab (1 in 200) to the sections. Incubate for 1 h at RT. *(Note: from this step forward, all steps should be performed in the dark)*
6. Wash slides with PBS and add the 2nd primary Ab overnight at 4°C.
7. Add the secondary Ab (1/200) for 1 hour after washing the sections thoroughly with PBS.
8. Wash sections and add Hoechst (1/200) for 15 min.
9. Wash slides well and mount with DAKO fluorescent mounting medium.
    1. *(Note: if only use 1 Ab, apply steps 1-5 and then 8 and 9)*