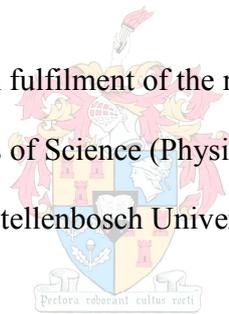


Hypothalamic-pituitary-adrenal-axis vs. the sympatho-adrenal medullary system in the acute response to psychological stress

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Declaration

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Abstract

The hypothalamic-pituitary-adrenal-(HPA) axis has long been closely associated with psychological stress-induced activation of the adrenal cortex and subsequent glucocorticoid production. Another, less known peripheral limb of the psychological stress response, is the sympatho adrenal medullary pathway.

We hypothesized that the sympatho-adrenal medullary system constitutes the primary response to acute psychological stress, with the HPA-axis functioning as a secondary response. We tested our hypothesis by manipulating a model of acute mild psychological stress (restraint) by blocking IL-6, a valuable constituent of the sympatho-adrenal medullary system.

Serum corticosterone concentration increased in response to stress (7 ± 3 vs. 57 ± 4 ng/ml; $P < 0.0001$), a response attenuated when IL-6 was blocked (17 ± 7 ng/ml). Stress increased pituitary mass only when IL-6 was blocked (38 ± 3 vs. 65 ± 6 mg; $P < 0.001$). Stress increased left adrenal mass only in the presence of IL-6 (34 ± 1 vs. 73 ± 8 mg; $P < 0.00001$). Stress did not influence the circulating levels of TNF- α , IL-1 β or IL-6 significantly. IL-1 β and TNF- α concentrations in the unstressed rats were lower when IL-6 was blocked.

We then manipulated the stress model by administering *S. frutescens* extract to elucidate both the central and peripheral effects of acute *S. frutescens* administration on the psychological stress response.

Restraint caused decreases in hippocampal GR levels when compared to respective controls. *S. frutescens* administration and exposure to restraint synergistically decreased hippocampal GABA_AR levels. In addition, exposure to both stress and *S. frutescens* led to a noteworthy increase in pituitary mass ($P = 0.078$), as well as pituitary ACTH levels ($P < 0.01$). Similarly,

differences in circulating ACTH levels showed an effect of stress on ACTH secretion only in the presence *S. frutescens* ($P < 0.05$). Adrenal mass was significantly increased in *S. frutescens*-treated animals that were also exposed to restraint ($P < 0.05$). Adrenal levels of ACTH showed a reciprocal trend to pituitary and circulating ACTH levels. No statistically significant differences were seen in adrenal IL-6 content. However, marked increases in IL-6 levels were seen at this level with administration of *S. frutescens* stress exposure and a cumulative increase seen with both *S. frutescens*-treatment and stress exposure.

Hippocampal GABA_AR, pituitary mass, pituitary ACTH and circulating ACTH levels showed a similar trend towards a synergistic effect of *S. frutescens* and restraint in activation of the psychological stress response, while adrenal ACTH levels showed an inverse trend. Hippocampal GR did not show any effect of stress or *S. frutescens*-treatment.

The results from these two experiments indicate that the sympatho-adrenal medullary system constitutes the primary response to acute mild psychological stress and that the HPA-axis is only activated during an exacerbated stress response or when the sympatho-adrenal medullary contribution is inadequate. Furthermore, the acute administration of *S. frutescens* possibly led to a functional shift in GABAergic function, resulting in activation of the stress response. The anecdotal reports of a “docile” effect of *S. frutescens* most likely results from activation of the mesolimbic dopaminergic system by the hippocampus and amygdala. These results have dramatic consequence in GABA-based anxiety-treatments.

Opsomming

Die hipotalamo-pituitêre-adrenale (HPA)-as is lank bekend as 'n primêre rolspeler in die respons op emosionele stres en daaropvolgende glukokortikoïed produksie. 'n Ander, minder bekende arm van die sielkundige stres respons is die simpatiese bynier-medulla-sisteem.

Ons hipotese was dat die laasgenoemde simpatiese bynier-medulla-sisteem die primêre respons tot sielkundige stres behartig terwyl die HPA-as 'n sekondêre respons bied. Ons het ons hipotese getoets deur die manipulerings van 'n beproefde stres model waar ons IL-6, 'n waardevolle rolspeler in die simpatiese bynier-medulla-sisteem, onderdruk het.

In respons op stress, het serum kortikosteroon konsentrasies toegeneem slegs in die teenwoordigheid van IL-6 (7 ± 3 vs. 57 ± 4 ng/ml; $P < 0.0001$), maar nie wanneer IL-6 onderdruk is nie (17 ± 7 ng/ml). Stres het 'n verhoging in hipofise massa teweeggebring slegs tydens die onderdrukking van IL-6 (38 ± 3 vs. 65 ± 6 mg; $P < 0.001$). Stres het ook linker-byniermassa verhoog slegs wanneer voldoende IL-6 beskikbaar was (34 ± 1 vs. 73 ± 8 mg; $P < 0.00001$). Stres alleen het geen invloed gehad op serum IL-1 β , IL-6 of TNF- α nie, maar die onderdrukking van IL-6 het wel 'n inhiberende effek op basale IL-1 β en TNF- α gehad.

Daarna het ons weer eens die stresmodel manipuleer deur die rotte 'n *S. frutescens* ekstrakt te gee in 'n poging om beide die sentrale en perifere effekte daarvan op die sielkundige stres respons te evalueer.

Stres alleen het gelei tot 'n afname in GR terwyl 'n kombinasie van stres en *S. frutescens* administrasie tot 'n afname in GABA_AR α 1 in die hippokampus gelei het. Hierdie kombinasie het ook tot 'n merkwaardige toename in hipofise massa ($P = 0.078$) sowel as ACTH-inhoud van

die hipofise ($P < 0.01$) gelei. 'n Soortgelyke patroon is waargeneem betreffende sirkulerende ACTH en byniermassa met $P < 0.05$ vir elk. Bynier ACTH inhoud, aan die ander kant, het 'n omgekeerd eweredige verhouding met ACTH in die hipofise en in sirkulasie getoon. Bynier IL-6 inhoud het geen statisties beduidende verskille getoon nie, maar 'n merkwaardige verhoging is weereens gesien met 'n kombinasie van stres en *S. frutescens* administrasie.

Die soortgelyke tendens wat waargeneem word in $GABA_A$ R in die hippokampus, asook hipofise- en sirkulerende ACTH vlakke, en dui op 'n samewerkende rol van stres en *S. frutescens* in die aktivering van die sielkundige stres respons. GR in die hippokampus toon geen veranderinge nie. Die resultate van die twee eksperimente dui op 'n primêre rol van die simpatiese bynier-medulla-sisteem in die respons op 'n akute stressor en dat die HPA-as net geaktiveer word tydens 'n ooreiste stres reaksie of indien die simpatiese bynier-medulla-sisteem onderdruk word. Die waargenome "verdownings"-effek van *S. frutescens* word moontlik deur aktivering van die mesolimbiese dopamien pad deur die hippokampus en amigdala bewerkstellig. Die resultate mag ook lei tot die heroorweging van GABA-gebaseerde angs medikasies.

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Abbreviations

11 β -HSD	11 beta-Hydroxysteroid dehydrogenase
A	antibody
ACTH	adrenocorticotropin hormone
ANOVA	analysis of variance
AVP	adenosine vasopressin
BDNF	brain derived neurotrophic factor
BNST	bed nucleus of the stria terminalis
CA	control antibody
CBG	corticosteroid binding globulin
CNS	central nervous system
CP	control placebo
CRH	corticotrophin-releasing factor
CSF	cerebrospinal fluid
CSu	control <i>S. frutescens</i>
cytGR	cytosolic glucocorticoid receptor
EC-CBr	endocannabinoid-cannabinoid receptors
EIA	enzyme-immune-assay
ELISA	enzyme-linked immunosorbent assay
FSH	follicle stimulating hormone
GABA	gamma-aminobutyric acid
GABA _A R	gamma-aminobutyric acid receptor A

GABA _B R	gamma-aminobutyric acid receptor B
GABA _C R	gamma-aminobutyric acid receptor C
GABA _A R α 1	gamma-aminobutyric acid receptor A subunit alpha 1
GAD	L-glutamic acid decarboxylase
G α S	G-protein subunit alpha-S
G $\beta\gamma$	G-protein subunit beta-gamma
GC	glucocorticoid
GH	growth hormone
Glu	glutamate
GnRH	gonadotropin-releasing hormone
HPA-axis	hypothalamo-pituitary-adrenal axis
HPG	hypothalamo-pituitary-gonadal
HRP	horseradish peroxidase
IEG	immediate early gene
IL	interleukin
i.p	intra peritoneal
LH	luteinising hormone
LNGFR	low-affinity nerve growth factor receptor
LPS	lipopolysaccharides
mbGR	membrane glucocorticoid receptor
ME	median eminence
MHC	major histocompatibility complex
MR	mineralocorticoid receptor

NIH	national institute of health
nNOS	neuronal nitric oxide synthase
NPY	neuropeptide Y
PBS	phosphate buffered saline
PKA	protein kinase A
POMC	pro-opiomelanocortin
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus
RT	room temperature
SA	stress antibody
SD	standard deviation
SEM	standard error of the mean
SNS	sympathetic nervous system
SP	stress placebo
SSu	stress <i>S. frutescens</i>
Su	<i>Sutherlandia frutescens</i>
<i>S. frutescens</i>	<i>Sutherlandia frutescens</i>
TMB	tetramethylbenzidine

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Chapter 1

Introduction

An organism perceives its environment along with potential threats through integrating sensory signals within the central nervous system. Potential threats (stressors) are perceived and evoke an intricate series of events known as the psychological stress response, leading to a neuroendocrine and immune reaction. Complex cognitive processes such as learning, memory and emotional processing serve to either keep an organism out of harm's way, or if need be, to fight off or flee from potential threats. Ineffective resolution of the stress response could however be responsible for causing a number of chronic ailments such as anxiety, depression and post-traumatic stress disorder (PTSD) (Miller *et al.*, 2005), as well as worsening other lifestyle associated diseases such as coronary heart disease (Vaccarino *et al.*, 2007) and other inflammatory-related ailments (Raison *et al.*, 2006). With rising suicide levels, related primarily to depression and other anxiety related disorders, it is imperative to investigate the factors involved in the proper resolution of the stress response.

The original concept of stress was defined as a physical strain eliciting an opposing force that would restore the system to an unstressed state. Canadian endocrinologist, Hans Hugo Bruno Selye, redefined the concept to “a state resulting in a nonspecific response of the body to any demand upon it” (Selye, 1975). The term “nonspecific” referred to a series of common responses which occur independently of the nature of the stressor. Building on the work of Cannon in the early 1900's who coined such phrases as “homeostasis” and the “fight or flight” response (Cannon, 1929), Selye proposed three universal stages of coping with a stressor and called it the General Adaptation Syndrome (Selye, 1976). The first stage, referred to as the “alarm reaction”, was synonymous to Cannon's “fight or flight” response

and associated with the sympatho-adrenal medullary system. This stage was followed by an acute adaptive stage associated with resistance to the stressor, which in the face of a persistent stressor, eventually leads to an exhaustion stage and the death of the organism. During Selye's early experiments on rats he noticed the enlargement of the adrenal glands after systemic injection with formalin- this eventually led to the discovery of the hypothalamic-pituitary-adrenocortical-(HPA) axis. This neuroendocrine pathway, along with the sympatho-adrenal medullary system, has become the two signaling pathways most closely linked to the stress response in mammals (Lopez *et al.*, 1999). Both these peripheral limbs of the stress response ultimately end in adrenal stimulation. Steroid secretion (glucocorticoids) from the adrenal cortex contributes to the resistance stage but may also contribute to the pathophysiology and pathogenesis of mood and anxiety disorders. Furthermore, inappropriate or chronic hyper-activation of the stress system may lead to downstream adrenal desensitization and eventual adrenal burnout that could in turn cause a variety of inflammatory related diseases, including coronary heart disease and stroke, and ultimately premature death.

The original dogma of non-specificity was modified by Chrousos and Gold (1992) who proposed that only above a certain threshold, would a stressor elicit a non-specific response. It was only in 1998 that Selye's dogma of non-specificity was put to experimental testing; the non-specific response to any given stressor could not be confirmed by further studies (Pacak *et al.*, 1998). This however did not prevent the acceptance of the concept of a general stress response by scientific literature. Within this framework of a general response, stress is defined as an intricate series of events, consisting of a stimulus (stressor), which precedes a reaction in the brain (stress perception), and activates a central nervous system (CNS) reaction (stress system), leading to a neuroendocrine response (Dhabhar and McEwen, 1999).

Studies done using immediate early gene (IEG) expression as a marker for neuronal activation have identified predominantly the prefrontal cortex and hippocampus, as well as several hypothalamic and amygdaloid nuclei, as being activated by psychological stress (Herman and Cullinan, 1997). Since these structures are activated regardless of the type of stressor (physical e.g. swimming/foot shock, or psychological e.g. immobilisation) applied, it is accepted that they form part of a “general” stress response. There is however a certain degree of specificity involved based on the different afferent inputs stimulating the response. Evidence therefore points to the presence of both a general and a stimulus-specific response, suggesting that the brain reacts to stress in a complex, coordinated manner and that this response necessitates activation of sensory, motor, autonomic, cognitive and emotional structures (Cullinan *et al.*, 1995, Li *et al.*, 1996, Campeau and Watson, 1997). The psychological stress response also involves other systems beside the central nervous system such as the endocrine, immune and peripheral nervous systems. It is therefore not surprising that links with these peripheral systems form the foundation for many of the adaptive and maladaptive responses of organisms to stress.

Different types of stress have been classified based on their duration and nature. Acute stress is defined as lasting no more than minutes to hours while chronic stress lasts from days or months, to years. Acute stress has been implicated in an increased release of pro-inflammatory cytokines such as interleukin-6 (IL-6), tumour necrosis factor (TNF)- α and interleukin-1 β (IL-1 β), whereas chronic stress is immunosuppressive (Elenkov and Chrousos, 1999b, a). Distinction between physical (local or systemic) stressors and psychological (progressive) stressors can also be made. The primary disparity between physical and psychological stressors is the central pathways that are activated during stress signaling and regulation. Physical stress signals reach the hypothalamus directly *via* the brainstem, while psychological stress signals require interpretation by higher structures of the limbic system

and frontal cortex. Psychological stress, as opposed to more physiological stressors, is a bi-dimensional concept with psychological aspects such as predictability, perception, control, and coping as well as physiological aspects involving different brain regions and neuroendocrine circuits activated by stress. The relationships between these systems are complex and incompletely understood (Lopez et al., 1999), but elucidation of these associations and bi-directional communication is needed to increase treatment options and improve long-term monitoring or preventative strategies. The aim of this thesis was to clarify interactions between role players in the context of stress, by manipulation of the stress response in an established *in vivo* rodent model. However, before attempting this, the basic understanding of role players is required. Therefore, in the next section, a review of the literature on these organs and systems is provided, in the context of acute stress.

Chapter 2

Literature review

This chapter will provide an overview of the literature pertaining to the acute stress response in terms of stress perception and central processing, signal transduction to effector organs, effector actions and role players in the regulation of this complex physiological response.

2.1 Stress Perception and Processing

Mechanisms of stress perception and processing will be discussed in terms of the limbic system, which consist of the amygdala, hippocampus, thalamus and hypothalamus, as well as neurotransmitters such as γ -aminobutyric acid (GABA) and glutamate.

2.1.1 The Limbic System

The stress-activated pathways responsible for the translation of stimuli into the final integrated response at the level of the hypothalamus are currently incompletely understood. It has been suggested that physiological stress is projected directly to the hypothalamus, most likely *via* the brainstem, while psychological stress or “progressive” stressors that usually require interpretation or modulation based on past experience may be relayed through the limbic-forebrain circuits (Herman and Cullinan, 1997). The limbic system forms a link between the higher and more complex mental activities of the cerebral cortex, and the lower or more basic functions such as heartbeat and breathing, which are regulated by the autonomic centers of the medulla. Anatomically, the limbic areas border the innermost sides of the cortex and are situated around the brainstem. The limbic system is involved in instinctive behavior and long-term memory. Nerve fibers connect components of this system,

such as the amygdala, hippocampus, thalamus and hypothalamus to other areas in the brain (Fig. 2.1), predominantly the lower frontal cortex, with its functions in anticipation, reward and decision making (Carter, 2009).

The thalamus is a pre-processing and communication centre from where all sensory information, with the exception of smell, is directed to the neocortical areas (“thinking brain”) *via* the mesolimbic pathway for processing in order to achieve conscious awareness. This dopaminergic pathway begins in the ventral tegmental area of the midbrain and connects to the limbic system *via* the nucleus accumbens, the amygdala, and the hippocampus as well as to the medial prefrontal cortex. It is known to be involved in modulating behavioral responses to stimuli that activate feelings of reward and motivation (Tisch *et al.*, 2004). The signal is subsequently relayed to the amygdala (“emotional brain”) for quick assessment and generation of emotional reactions (Kaplan *et al.*, 2007).

The amygdala sends impulses to the hypothalamus for activation of the sympathetic nervous system and subsequent HPA axis activation. During processing of stimuli perceived to be potentially threatening however, the thalamus bypasses the cortex and routes the signal directly to the amygdala, which is the trigger point for the primitive fight-or-flight response (labeled the amygdala hijack) (Goleman, 1996). The amygdala contains different regions called nuclei, which are distinct collections of the cell bodies (soma) from neurons that project signals to an array of other areas. In the amygdala these nuclei produce different responses to fear, e.g. the central nucleus initiates a freezing response, while the basal nucleus initiates the flight response (Rasia-Filho *et al.*, 2000). Activation of amygdaloid nuclei are influenced by sex hormones and therefore differ between males and females. Progesterone for example, has been shown to be a potent barbiturate-like ligand of the GABA receptor–chloride ion channel complex, which has potent sedative and hypnotic effects (Majewska *et al.*, 1986). This sexual dimorphism could explain certain behavioral differences between

males and females, especially during the psychological stress response (Rasia-Filho et al., 2000) such as the “tend-and-befriend” behavioural pattern seen in females as opposed to the “fight or flight” seen in males (Taylor *et al.*, 2000). Interestingly, classical conditioning is facilitated by psychological stress in males, while being impaired in females in an *in vivo* model where rats were exposed to intermittent restraint stress (Conrad *et al.*, 2004).

The amygdala in turn projects signals to the hypothalamus through the bed nucleus of the stria terminalis (BNST), which forms part of the “extended amygdala”. The parvocellular neurons of the paraventricular nucleus (PVN) in the hypothalamus represent the final common path for the integration of the vast array of descending circuits activated by the stress response in the brain (Lopez et al., 1999). In addition, experiments performed by Cullinan *et al.* (1993) and Herman *et al.* (1992; 1994) also elucidated an alternative signaling pathway from the hippocampus, which also receives input from the amygdala directly, to the BNST and from there to the PVN. The hippocampus is located along the inside of the parahippocampal gyrus. Gyri are the bulges of the brain which are usually surrounded by one or more sulci (folds stretching towards the centre of the brain) (Carter, 2009). The latter are used as orientation landmarks when investigating the brain. The hippocampus also interlocks with another crease known as the dentate gyrus, which collectively forms the hippocampal-dentate complex. Even though this complex forms part of the cerebral cortex, it consists of only one to three layers of cells compared to the usual six layers of the more sophisticated regions of the cortex (Carter, 2009). The hippocampus is involved in establishing memory, by encoding information that is consciously perceived to form memories. The hippocampus also relays stored information back to the amygdala and other parts of the cortex to elicit confirmation or modification of the initial response to psychological stress. It is noteworthy that there are more projections from the amygdala to the hippocampus than *vice versa*, indicating a flow of information from the amygdala to the hypothalamus *via* the

hippocampus. These hippocampal inputs to the hypothalamus are primarily mediated by GABAergic neurons (neurons containing the neurotransmitter GABA) and are inhibitory in nature, suggesting a regulatory role for the hippocampus in the psychological stress response (Jacobson and Sapolsky, 1991). The main hippocampal projections are from the lateral nucleus to the entorhinal cortex, an area of the cortex from which the hippocampus receives most sensory input. The hippocampus, along with being involved in spatial learning, also plays a vital role in psychological stress regulation by propagation of inhibitory neurotransmissions, thereby shifting neuronal output from sympathetic to parasympathetic. The best characterized inhibitory outputs from the hippocampus are conveyed through the ventral subiculum and the BNST-PVN (Lopez et al., 1999), the latter consisting almost entirely of GABAergic neurons (Ferraguti *et al.*, 1990). This function will be discussed in more detail in section 2.3.3.

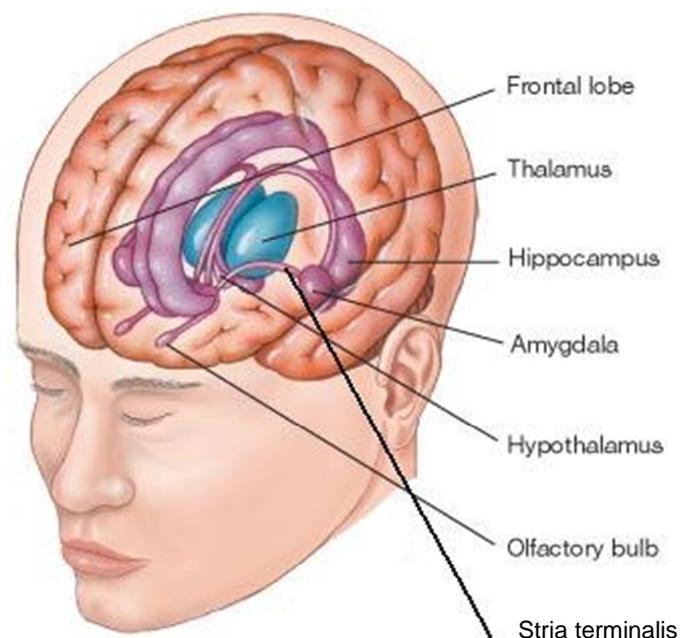


Figure 2.1. Relevant areas of the limbic system adapted from Essential_Survival, (2010).

2.1.2 Neurotransmitters

Neurotransmitters are chemicals that allow signals to travel between neurons and from neurons to other effector cells. There are numerous types of neurotransmitters: some contain only acetylcholine, while another group - known as monoamines - includes dopamine, serotonin (5-hydroxytryptamine or 5-HT), noradrenaline and histamine. A third group is comprised solely of amino acids such as glycine, aspartate, glutamate and GABA. A particular neurotransmitter can either excite a receiving cell, helping to depolarize the axon and convey a nerve impulse, or inhibit it by down-regulating depolarization (Carter, 2009).

GABA is the most abundant inhibitory neurotransmitter in the mammalian central nervous system, occurring in up to 40% of all synapses (Uhart *et al.*, 2004). GABA concentrations in the brain can be between 200 and 1000 times higher than that of monoamine or acetylcholine neurotransmitters, with only glutamate, the major excitatory neurotransmitter, occurring in higher concentrations. Together GABA and glutamate maintain the homeostatic activity of neuronal transmission by balancing the inhibition and excitation of neuronal circuits. Both neurotransmitters are synthesized in the brain from the Krebs cycle associated citric acid molecule alpha-ketoglutarate in a process known as the GABA shunt (Fig 2.2). Even though GABA can cross the blood brain barrier (Kuriyama and Sze, 1971), it is also synthesized from glutamate by an enzyme called L-glutamic acid decarboxylase (GAD). Pyridoxal phosphate, a vitamin B6 derivative, is a cofactor in the synthesis of GABA from glutamate, explaining the incidence of convulsive seizures in patients with a vitamin B6 deficiency (Bayoumi *et al.*, 1972).

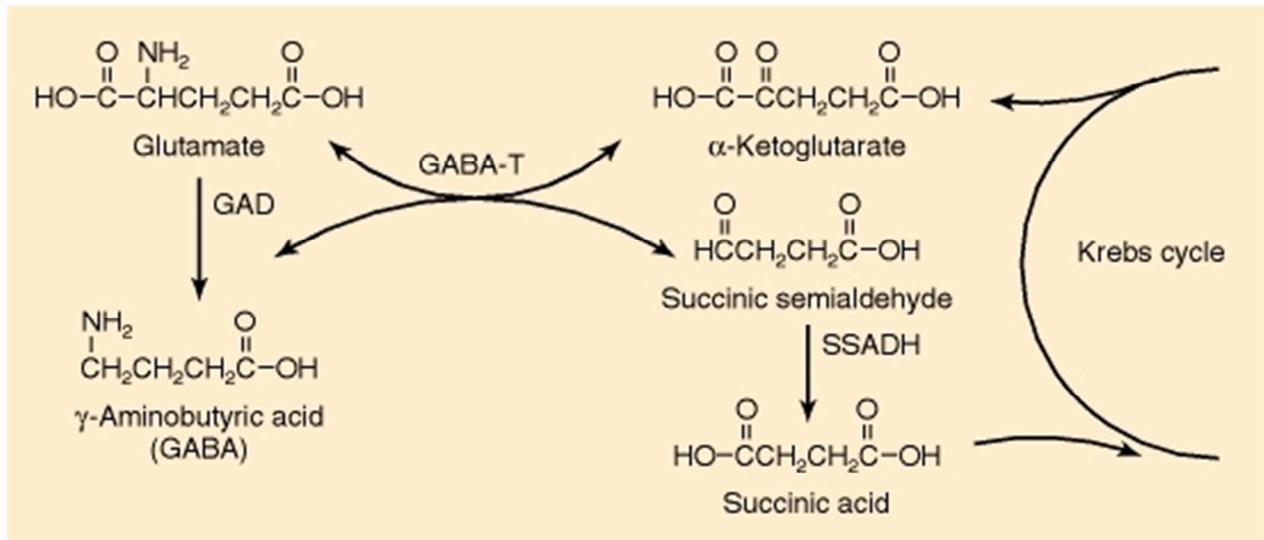


Figure 2.2. GABA shunt from the Krebs cycle (Olsen and DeLorey, 1999).

GABA predominantly acts at inhibitory synapses in the substantia nigra and globus pallidus nuclei of the basal ganglia, the hippocampus and the hypothalamus. It performs its inhibitory function by binding to specific transmembrane receptors in the plasma membrane of both the pre- and post-synaptic neurons, causing the opening of ion-channels. A subsequent influx of negatively charged chloride ions along with an efflux of positively charged potassium ions result in a state of hyperpolarization of the neuronal trans-membrane potential, inhibiting the action potential responsible for signal transduction (Carter, 2009). The diverse set of membrane bound receptors influenced by GABA can be divided into two major groups: ionotropic receptors that are ligand-gated ion channels (GABA_AR GABA_CR), and metabotropic receptors that are G-protein coupled (GABA_BR) and which fulfill their function through second messenger systems (Bormann, 2000, Chebib and Johnston, 2000). Work done by Le Novere and Changeux demonstrated that the ionotropic receptors were part of a nicotinic receptor superfamily that also binds nicotinic acetylcholine, as well as serotonin. GABA_AR and GABA_CR can further be subdivided on account of their physiological and pharmacological properties as well as the fact that they form endogenous heteromeric and homomeric receptors respectively, consisting of five subunits each (Le Novere and

Changeux, 2001). These five protein subunits are assembled around a central pore that comprises the actual chloride ion channel (Johnston, 2005). GABA_AR is responsible for most of the GABAergic signal transduction in the brain and in the context of stress, it functions to down-regulate the psychological stress response by inhibition of hypothalamic PVN and anterior pituitary neurons (Myerhofer *et al.*, 2001). GABA_AR, however, has been shown to be down-regulated in response to acute immobilisation stress (Zhang *et al.*, 1990). Furthermore, other types of acute psychological stress, such as social isolation, decrease the function of the GABA_AR receptor complex (Magarinos *et al.*, 1996). GABA_AR and GABA_BR receptors are also present in the adrenal gland, where they regulate sympathetic-stimulated cortical steroidogenesis (Mishunina and Kononenko, 2002). This regulatory function of GABA will be further discussed in section 2.3.5. Since GABA_AR is present in both the central and peripheral nervous system, it is a useful marker of psychological stress inhibition throughout the body. In general, changes in receptor levels probably provide a more accurate reflection of GABA function in target tissue since GABA has a relatively short half-life of approximately 1.9 minutes (Enna and Snyder, 1975). Exposure to chronic restraint stress led to a significant reduction in GABA_AR receptor binding in the prefrontal cortex. Changes in specific binding of GABA to its receptors were not seen in the cerebellum, caudate-putamen, or hippocampus however, suggesting that the effects of chronic stress may be regionally specific (Gruen *et al.*, 1995). Increases in GABA_AR levels observed in response to an acute forced swimming test suggest an important role for GABA along with its receptor in the modulation of the acute psychological stress response (Uhart *et al.*, 2004).

2.2 Stress signal transduction

Signal transduction of stress signals follow two major pathways in order to reach the main peripheral stress organ a.k.a. the adrenal gland. As discussed above, the hypothalamic PVN acts as the final converging point of monosynaptic and multi-synaptic inputs from several areas in the brain. This group of parvocellular neuronal cell bodies produces both hormonal and neuronal signals that projects to the pituitary gland and the brainstem respectively. as a result, dissociation between the ACTH-mediated neuroendocrine limb (HPA) and the non-ACTH mediated sympathetic limb (sympatho-adrenal medullary system) of the psychological stress signal transduction occurs.

2.2.1 The Hypothalamic-Pituitary-Adrenal (HPA)-axis

The HPA-axis comprises of both neuronal circuits and endocrine components, with feed-forward and feedback interaction between components, and therefore represents a classic “neuroendocrine” circuit (Ehrhart-Bornstein *et al.*, 1991, Herman and Cullinan, 1997, Lopez *et al.*, 1999). It is at the level of hypothalamus where the stress response is transduced from a neuronal to an endocrine pathway. The cells of the PVN express corticotrophin-releasing factor (CRH) in response to physiological stress signals, originating from the brainstem, as well as psychological stress signals from the thalamus (Cullinan *et al.*, 1996). CRH is released from the median eminence into the hypophyseal portal circulation to reach the anterior pituitary. During ACTH-mediated stress signal transduction the pituitary gland or “master gland” is stimulated by CRH to secrete adrenocorticotrophic hormone (ACTH) from its pro-opiomelanocortin (POMC) producing cells (corticotrophs) through POMC precursor molecule processing in the anterior pituitary (Feldman *et al.*, 1995). ACTH travels through the systemic circulation and binds to its receptors in the adrenal cortex. These receptors,

upon ligand binding, undergo conformational changes that stimulate the enzyme adenylyl cyclase, leading to an increase in intracellular cAMP and subsequent activation of protein kinase A. This ultimately results in stimulation of steroidogenesis in the zona fasciculata of the adrenal cortex. As a result of acutely increased steroidogenesis the adrenal gland undergoes certain morphological changes, such as cellular hypertrophy, hyperplasia and decreased number of liposomes while chronically augmented steroidogenesis could result in hypervascularisation, along with increased density of mitochondria and smooth endoplasmic reticulum, (von Euler, 1967). Liposomes store cholesterol, the substrate of glucocorticoid biosynthesis (Bornstein *et al.*, 1992, Nussdorfer and Gottardo, 1998). Glucocorticoid secretion from the adrenal cortex is the final product of the psychological stress response (discussed in further detail at the end of the section).

2.2.2 The Sympatho-adrenal medullary system

The sympatho-adrenal medullary system, with the brain stem and hypothalamus as central components, relies on the transduction of a signal from the central to the sympathetic nervous system. The majority of these sympathetic nerve fibers have been demonstrated to be cholinergic preganglionic sympathetic fibers arising predominantly from the third thoracic and second lumbar vertebra/regions of the spinal cord (Hollinshead, 1937). Relatively more modern retrograde fiber tracing techniques verified these claims but also produced morphological evidence of both, preganglionic and postganglionic, sympathetic and parasympathetic, innervations along with an afferent baroreceptor component innervating the adrenal glands (Kesse *et al.*, 1988, Afework, 1989, Coupland *et al.*, 1989). The majority of nerves penetrate the adrenal capsule where they branch to form an extensive subcapsular network. The possibility of an integrated neural regulation of both the cortex and medulla has led to a unified approach to understanding adrenal function. The cells of the adrenal

gland receive both extrinsic as well as intrinsic innervations, the latter consisting of ganglion cells scattered throughout the cortex and medulla (Coupland, 1965). Ultrastructural studies performed in the nineteen seventies revealed the existence of synaptic terminals adjacent to cortical cells (Unsicker, 1971, Robinson *et al.*, 1977). The ganglion cells are said to originate from sympathetic neurons that differentiated from the neuronal crest cells which migrated into the cortical zonas (Pelto-Huikko *et al.*, 1985). Even though traditionally only the adrenal medulla was associated with innervation, evidence now suggests the presence of a smaller quantity of branched neural fibers also distributed into the cortex (Parker *et al.*, 1993). Signals travel through the splanchnic nerves to the adrenal medullae (Goldstein, 1995), and are mainly cholinergic and peptidergic, storing the catecholamines dopamine, epinephrine and nor-epinephrine along with a variety of neuropeptides such as neuropeptide Y (NPY) and CRH. Catecholamine secretion is mediated by nicotinic cholinergic receptors located in the membranes of medullary chromaffin cells (Parker *et al.*, 1993). Intra-adrenal communication between the two ontogenetically different regions (regions originating from different embryonic germ cell lines) of the adrenal gland, are mediated by a variety of secretory products. *In situ* and *in vitro* studies have shown epinephrine and nor-epinephrine, the main adrenomedullary secretory products, to stimulate adrenocortical function by augmentation of the transcriptional activity of several steroidogenic factors and enzymes (Ehrhart-Bornstein *et al.*, 1991, Guse-Behling *et al.*, 1992). Probably the most significant splanchnic contribution to adrenocortical steroidogenesis is mediated through these catecholamines and other neuropeptides released from adrenomedullary chromaffin cells in large amounts. Previous studies performed by our group also demonstrated the IL-6 dependent nature of psychological stress-associated corticosterone release. This finding is supported by literature where mRNA analysis of IL-6 and IL-6 receptor gene expression indicated a para- and/or autocrine function of IL-6 in adrenal medulla regulation (Gadient *et al.*, 1995). It is therefore plausible to

assume that IL-6 plays a role in the adrenal activation by sympathetic nerves. This role of IL-6 will be further discussed in section 2.3.6.

The influence of the sympatho-adrenomedullary system on the psychological stress response can also be demonstrated by its ability to increase adrenocortical sensitivity to ACTH. Edwards *et al.*, 1998 showed that stimulation of splanchnic nerves resulted in enhanced production of glucocorticoids in response to a standardized ACTH stimulus, while splanchnic lesions decreased adrenocortical sensitivity to ACTH. Studies also showed that perfused pig adrenal glands with intact splanchnic innervations could elicit steroidogenesis upon electrical activation in the absence of pituitary-derived ACTH (Ehrhart-Bornstein *et al.*, 1991, Ehrhart-Bornstein *et al.*, 1994, Ehrhart-Bornstein *et al.*, 1995). Furthermore, the sympatho-adrenomedullary system seems to contribute to compensatory growth signals in remaining adrenal glands after unilateral adrenalectomy (Dallman *et al.*, 1976). The adrenal gland can therefore be described as a modified sympathetic splanchnic ganglion surrounded by a steroidogenic endocrine gland, and appears to form another link between nerve and hormone systems in the integrated neuro-endocrine stress response.

Morphologic characteristics of the adrenal gland provide clues as to how the medullary secretions reach cortical cells. Contrary to popular belief the cortex and medulla are not clearly separated but rather intertwined with adrenomedullary chromaffin cells found in all three cortical zones and cells from all these zones found within the medulla (Gallo-Payet *et al.*, 1987). Chromaffin cells in the cortex either form ray-like structures, radiating outwards, or small chromaffin islets, while some are simply dispersed throughout the zona fasciculata and reticularis, surrounded by steroid-producing cells. In the capsular region of the zona glomerulosa, chromaffin cells often form subcapsular nests (Fortak and Kmiec, 1968). On the other hand, islets of cortical cells are found within and bordering adrenomedullary

chromaffin tissue (Bornstein *et al.*, 1994). Ultrastructural analysis of contact areas between cortical and medullary cells throughout the different cortical zones show no separation by connective tissue or interstitial fluid and allow for extensive paracrine interaction (Hoheisel *et al.*, 1998). Studies performed in cat adrenals demonstrated additional transport mechanisms of adrenomedullary secretory products through interstitial fluid and lymphatics. Except for catecholamines, which enter the blood vessels directly, other larger secretory molecules such as neuropeptides cross into and out of the lymph to reach the adrenocortical cells. Catecholamines, due to its swift entry into circulation, can only influence cortical cells in direct contact with the catecholamine-producing chromaffin cells to secrete corticosterone (Carmichael *et al.*, 1990).

Once in circulation, catecholamines prepare the body for the fight or flight response by binding to β -adrenergic receptors on a vast array of target tissues. Some of these preparations include redirecting blood flow from organs to skeletal muscle, increasing heart rate, dilating pupils, along with numerous other effects that serve to improve the organism's chances of surviving stressful events (Sherwood *et al.*, 2004).

The function of both above mentioned systems are crucial for an integrative stress response and their regulation is therefore a complex, adaptive process characterized by compensation by one pathway in the event of alternative pathway inhibition. Nevertheless, upon activation, both pathways eventually lead to downstream glucocorticoid production and release from the adrenal cortex.

2.2.3 Glucocorticoids

Glucocorticoids are steroid hormones that derive their name from their primary function, which is to increase blood glucose levels, as well as their site of production, i.e. the adrenal

cortex. The predominant glucocorticoid in humans is cortisol, while corticosterone is most abundant in rodents. An average person secretes between 10 to 20 mg cortisol per day during his/her circadian rhythm, which is also under the control of pituitary ACTH (Katzung, 2004, Goodman *et al.*, 2006). Under normal circumstances, 90% of plasma cortisol is bound to circulatory proteins called corticosteroid binding globulin (CBG) while the remaining 10% is either free or loosely bound to albumin, and thus accessible for glucocorticoid receptor binding.

Glucocorticoids perform a wide variety of functions through glucocorticoid receptor binding, including metabolic (glucogenolysis and gluconeogenesis) as well as immune (suppression of cell-mediated and humoral immunity) regulation. The majority of these functions are achieved by genomic regulation, such as limiting the number of circulating neutrophils and macrophages by suppressing the production of pro-inflammatory cytokines. Glucocorticoids have been shown to induce apoptosis in lymphocytes (Seki *et al.*, 1998) and eosinophils (Schleimer and Bochner, 1994), and up-regulate phagocyte capacity to clear apoptotic neutrophils (Nittoh *et al.*, 1998). Another contribution of glucocorticoids to inflammatory resolution occurs through non-genomic regulation and involves a biosynthetic shift from inflammatory arachidonic acid toward anti-inflammatory endocannabinoids (Malcher-Lopes *et al.*, 2008). Glucocorticoids are also vital in the regulation of the reproductive and central nervous system (Lu *et al.*, 2004). This aspect was discussed already in terms of steroidogenesis in section 2.3.2. Maintenance of elevated plasma glucocorticoid levels during times of acute and chronic psychological stress requires a shift in substrate allocation away from anabolic steroids, such as testosterone, growth hormone (GH) and estrogen, toward glucocorticoid synthesis, which results in an inevitable reduction in other adrenal steroids. The pathways involved with the steroidogenic shift can be attributed to increases in pro-inflammatory mediators activated by the psychological stress response, particularly cytokines

produced within the adrenal gland. It is also speculated that activated lymphocytes within the adrenal medulla could trigger a major histocompatibility complex (MHC) class II-mediated cell death, class II being the dominant MHC molecule expressed on adrenomedullary androgen producing cells (Marx *et al.*, 1998). This shift, however, is crucial for survival when threatened by stress or illness. The stress regulatory role of glucocorticoids will be discussed in more detail in section 2.3.

2.3 Stress regulation

Both activation and termination of the adrenocortical stress response are vital to the adaptation and survival of the organism. It is known that chronic stress that coincides with elevated corticosterone levels is connected to various harmful effects such as adrenal burnout, which could lead to inflammatory related ailments such as diabetes and coronary heart disease. Different role players in the regulation of the response to stress will be discussed in the next section.

2.3.1 Glucocorticoid-associated systems

2.3.1.1. Traditional glucocorticoid negative feedback loop

Circulating glucocorticoids inhibit their own secretion through three mechanisms, namely a rate-sensitive fast feedback, an intermediate feedback and a delayed feedback (Keller-Wood and Dallman, 1984). The fast feedback occurs within minutes and is based on the rate of increase in glucocorticoid levels rather than the total systemic level. This mechanism is achieved in part by the binding of glucocorticoids to their specific receptors (glucocorticoid receptors) in the hypothalamus, which inhibits CRH secretion, while in other limbic structures such as the hippocampus, it down-regulates excitatory glutamatergic signals and increases inhibitory GABAergic signal transmission. Intermediate and delayed feedback

occurs over the course of hours to days and is based on the transcriptional suppression of CRH and POMC gene expression by translocated glucocorticoid receptor (GR) complexes. Even though a portion of the functions performed by glucocorticoids are mediated through G-coupled proteins, the predominant functional feedback route followed by glucocorticoids, involve GR.

2.3.1.2 *Glucocorticoid Receptors*

According to both functional and biochemical traits, two types of corticosteroid receptors are distinguished, and both are also present in the brain (de Kloet *et al.*, 1998b). Type I or mineralocorticoid receptor (MR) has high affinity for cortisol/corticosterone and is primarily found in the hippocampus and other limbic areas, whereas Type II, or low affinity glucocorticoid receptor (GR), is more widely distributed across the regions of the brain (Zhe *et al.*, 2008). In the rest of the body, MR and GR are expressed in almost every cell in the body and regulates genes controlling the development, metabolism, and immune response (Lu *et al.*, 2006).

These receptors mediate a variety of effects on neuronal excitability, neurochemistry and neuroplasticity (McEwen, 1999). MR, being described as a “high-affinity, low-capacity glucocorticoid receptor system”, is said to offer tonic inhibition of the axis during the nadir of the circadian rhythm (Funder, 1986, de Kloet *et al.*, 1998b, Lopez *et al.*, 1999). However, during the psychological stress response cortisol levels increase dramatically, leaving the MR saturated and the GR, being described as a “low-affinity, high-capacity” receptor, maintaining or facilitating the return to homeostasis.

In the context of stress regulation, MR has also been illustrated in the kidney where, due to the presence of an enzyme known as 11-beta hydroxysteroid dehydrogenase (11 β -HSD), it also has affinity for aldosterone. 11 β -HSD is part of an enzyme family which catalyze the

conversion of inert 11 keto-products (cortisone) to active cortisol, and *vice versa*. In the kidney, it inactivates cortisol to cortisone, enabling aldosterone to bind to the MR. 11 β -HSD is also found in the liver, adipose tissue and central nervous system where it contributes to glucocorticoid regulation (Seckl and Walker, 2001). Although the exact effect of stress on the GR is controversial, previous studies conducted by our group as well as other groups showed a marked reduction of the GR in response to acute and repeated psychological stress in both the brain and peripheral tissues such as the liver (Omrani *et al.*, 1980, Sapolsky *et al.*, 1984, Smith *et al.*, 2007).

2.3.1.3. *Stress and inflammation*

Acute psychological stress is associated with an increase in pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α (Maes *et al.*, 1998, Smith *et al.*, 2007) at different time points. These increases in pro-inflammatory cytokine levels are necessary for proper HPA-axis function on multiple levels. For example, IL-6 have been shown to contribute to the psychological stress-induced corticosterone release directly by stimulation of the adrenal gland (Path *et al.*, 1997, Franchimont *et al.*, 2000b), or upstream via activation of the HPA-axis (Mastorakos *et al.*, 1993, Zhou *et al.*, 1993). IL-6 is also associated with greater bioavailability of corticosterone through the inhibition of cortisol binding globulin, thereby enhancing the stress response (Bartalena *et al.*, 1993). Inflammatory cytokines such as IL-6 thus plays a pivotal role in strengthening the response to psychological stress.

One of the primary functions of glucocorticoids could be described as a mediator of inflammatory resolution; with binding of glucocorticoids to their specific cytoplasmic GR leads to the translocation of the GR to the nucleus where it acts as ligand-induced transcription factor (Caamano *et al.*, 2001). GR either binds directly to cognate response elements in target gene promoters, thereby regulating gene expression, or obstructs other

signaling pathways such as those involving nuclear factor $\kappa\beta$ (NF- $\kappa\beta$), an inducible transcription factor complex that regulates the expression of a range of genes involved in inflammatory immune responses (Smets *et al.*, 1999).

In contrast, *in vivo* studies also illustrated that a GR antagonist could not block the inhibition of CRH-induced ACTH secretion after an intravenous glucocorticoid injection (Hinz and Hirschelmann, 2000). The same measure of inhibitory effects was seen even after the blocking of gene transcription and protein synthesis *in vitro* (Dayanithi and Antoni, 1989). This points to the alternative non-genomic nature of the GR. Recent findings suggested that glucocorticoids induce the synthesis of arachidonic acid-derived endocannabinoids in the neuroendocrine cells of the hypothalamus. This lowers the availability of arachidonic acid for pro-inflammatory prostaglandin synthesis (Malcher-Lopes *et al.*, 2008). These non-genomic regulatory actions of glucocorticoids entail inhibition of various pathways by intracellular and/or membrane-bound receptors and are especially active in neurons of the central nervous system. The reduction in the inflammatory input marks the resolution of the psychological stress response and constitutes another more indirect contribution of glucocorticoids to stress termination as seen in Fig 2.3.

2.3.1.4. Neuronal regulation by glucocorticoids

In addition to the immune-regulatory functions of glucocorticoids in stress regulation, the limbic circuits of the amygdala and hippocampus, along with their neurotransmitters, are also regulated through non-genomic glucocorticoid action. Di *et al.* (2003) recently proposed that glucocorticoids elicit a rapid suppression of excitatory glutamatergic synaptic inputs while having an opposite effect on inhibitory GABAergic neuronal inputs to the neuroendocrine cells of the PVN in the hypothalamus. Glucocorticoids are therefore an important role player in the maintenance of a normal excitatory/inhibitory balance in the central nervous system.

This regulation is said to function through activation of a postsynaptic, membrane-associated receptor and the G-protein-dependent synthesis of retrograde endocannabinoid messengers (Di *et al.*, 2003, Di *et al.*, 2005). The pathways involved in this glucocorticoid-mediated endocannabinoid synthesis necessary for the opposing regulation of GABA and glutamate, are the $G_{\alpha S}$ G-protein subunit and the cAMP-dependent protein kinase (PKA) pathway (Malcher-Lopes *et al.*, 2008). In a study by Di *et al.* performed in 2003, they determined that glucocorticoids activate different G-protein signaling pathways in parallel, via the $G_{\alpha S}$ and $G_{\beta\gamma}$ subunits, and subsequent production of endocannabinoids and nitric oxide (NO) respectively. These retrograde messengers act in a synapse-specific manner to suppress excitatory synaptic inputs, as seen in the case of endocannabinoid-binding to its pre-synaptic receptor, and facilitate inhibitory synaptic inputs, in the case of NO (Di and Tasker, 2008). Therefore, glucocorticoid-induced shift in neuronal activity from excitatory (sympathetic) towards inhibitory (parasympathetic) signaling in, for example the hippocampus, could attenuate the psychological stress response at perceptual level and play a pivotal role in stress regulation (Fig. 2.3).

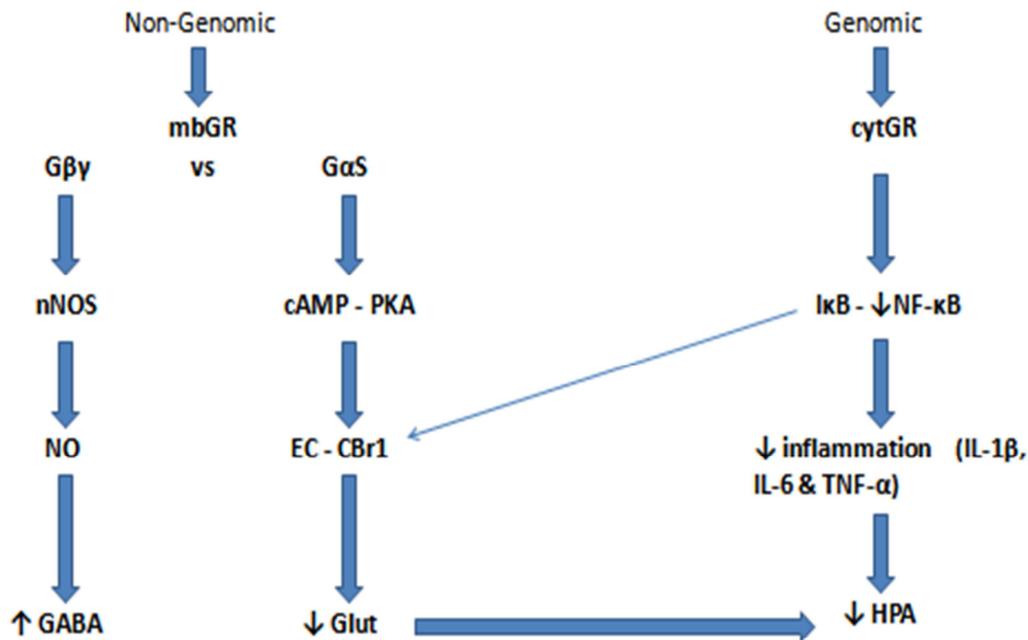


Figure 2.3. Glucocorticoid receptor mediated contribution to resolution of the psychological stress response can be divided into non-genomic and genomic functions. The non-genomic functions are mediated through membrane bound glucocorticoid receptors (mbGR) which consist of Gβγ and GαS proteins that activates neuronal nitric oxide synthase (nNOS) and the cAMP-PKA pathways to produce NO and cannabinoid receptor 1 (CB1) respectively. The increased intracellular NO concentrations within neurons causes increased GABA release while binding of endocannabinoids (EC) to CBr1 upregulates glutamatergic (Glu) neurotransmission. The increased GABAergic and decreased glutamatergic neurotransmission increases the inhibitory input to the psychological stress response. The genomic functions are mediated through cytosolic glucocorticoid receptors (cytGR) which activates inhibitor of kappaB (IκB), downregulating nuclear factor-κB (NF-κB) which is responsible for stimulating the transcription of inflammatory cytokines. The decreased cytokine levels signal the resolution of inflammation which is also a signal for the down-regulation of the psychological stress response.

2.3.2 Testosterone

Testosterone is a steroid hormone and the most important androgen secreted into the bloodstream. In mammals testosterone is primarily secreted by Leydig cells in the testes of males and the ovaries of females, while small amounts are secreted by the adrenal cortex. Testosterone is also the principle male sex hormone and a powerful anabolic steroid and is regulated by the Hypothalamic-Pituitary-Gonadal (HPG) axis (Selye, 1946). Gonadotropin-

releasing hormone (GnRH) is secreted by the hypothalamus which in turn stimulates the pituitary gland to release follicle stimulating hormone (FSH) and luteinising hormone (LH).

These latter two hormones stimulate the testis to synthesize testosterone. Increasing levels of testosterone act on the hypothalamus and pituitary through a negative feedback loop to inhibit the release of GnRH and FSH/LH respectively (Swerdloff *et al.*, 1992, Payne and O'Shaughnessy, 1996). The reproductive system undergoes significant inhibition during the adaptive response to psychological stress (Johnson *et al.*, 1992). Hans Selye proposed that chronic stress causes an exacerbated HPA axis response, while inhibiting the HPG-axis (Selye, 1939). Rat studies on the chronic response to immobilisation, immersion in cold water, and electrical foot shock have shown decreases in serum LH and testosterone 28 days after initiation of the stress protocols (Sapolsky and Krey, 1988, Norman and Smith, 1992), while human studies show marked negative effects of stress on various parameters of semen quality such as sperm concentration, motility and morphology (Moghissi and Wallach, 1983, Bents, 1985, Giblin *et al.*, 1988). These changes were attributed to lower levels of LH and testosterone (Tilbrook *et al.*, 1999). 11 beta-hydroxy-steroid dehydrogenase (11 β -HSD) in Leydig cells oxidatively inactivates corticosterone, decreasing its concentration, as mentioned earlier, thereby protecting cells against the suppressive effect of glucocorticoids on testosterone production (Monder *et al.*, 1994, Gao *et al.*, 1996). Even though numerous studies has been done in this field using different models of stress, few of them actually show correlations between increases in corticosterone and testosterone suppression, nor can it be confirmed that acute stress negatively affects sexual behavior (Retana-Marquez *et al.*, 2003). On the other hand, chronic stress have been implicated in increased plasma corticosterone levels in rats and hamsters, and increased cortisol levels in humans, while inhibiting the HPG axis (Collu *et al.*, 1984, Remes *et al.*, 1985, Gonzalez-Quijano *et al.*, 1991). Whether or not testosterone synthesis is significantly influenced by acute activation of the sympatho-adrenal

medullary system is unclear. The elucidation of this matter is necessary in order to inform on possible side-effects of stress, which should be considered in development of anti-anxiety remedies.

2.3.3 Hippocampal regulation

Higher limbic structures such as the hippocampus, and in particular the GABAergic neuronal connections of the BNST between the hippocampus and the hypothalamic CRH neurons, also play a role in the regulation of the HPA-axis (Herman *et al.*, 1992, Cullinan *et al.*, 1993, Herman *et al.*, 1994, Herman *et al.*, 1996). Studies using gross hippocampal lesions along this tract, as well as hippocampallectomies, have shown an up-regulation of CRH mRNA during increased circulating corticosterone and ACTH levels (Herman *et al.*, 1989). It appears that the results of these studies point to a decreased negative feedback as well as an increased basal drive. Evidence arising from these studies also point to a role for the hippocampus in maintaining the basal tone of the HPA-axis. The tonic inhibition of ACTH secretion from pro-opiomelanocytes in the hypothalamic PVN by the hippocampus is likely achieved through the binding of corticosterone to hippocampal GR (Herman *et al.*, 1989) as well as the indirect activation of inhibitory GABAergic neurons, mainly in the hippocampal CA1-3 regions (Jacobson and Sapolsky, 1991), by elevated corticosterone levels in response to stress. This is likely mediated *via* a negative feedback by glucocorticoid binding to G-coupled proteins located in the hippocampus and subsequent inhibitory neuronal inputs to the hypothalamic PVN (Herman *et al.*, 1989). Collectively these studies suggest a major role for the hippocampus in the modulation of basal as well as stress associated levels of downstream CRH and ACTH by reciprocal input to the thalamus and PVN of the hypothalamus.

2.3.4 GABAergic regulation

Of similar importance is the biochemical constituents involved in the circuitry between limbic and peripheral components, and their role in the regulation and termination of the stress response. Inhibitory hyper-polarization of central neurons prevents hyper-excitation and provides suitable down-regulation after stressful experiences. This balance between excitatory glutamate and inhibitory GABA is crucial for the maintenance of homeostasis and dysfunction or deficiency in either could lead to severely decreased quality of life and even premature death. Although GABAergic function in the cerebral cortex does not seem to be affected by an acute pain stress (Sherman and Gubhart, 1974), striatal pathways (the main neuronal pathways running from the forebrain to the basal ganglia) are very sensitive to acute stress experiences (Losada, 1988). A study where GABA, along with its synthesising and degradation enzyme activity were measured, showed a markedly decreased GABA turnover in the corpus striatum directly after one hour of immobilisation stress and an increase to levels above baseline three hours after cessation of stress exposure in a super-compensatory fashion (Yoneda *et al.*, 1983, Losada, 1988). Experimental animals displayed signs of hyper-excitability at time points during a depressed GABA activity phase, explaining the correlation between GABAergic activation and docile behavioral patterns. GABA contributes to stress resolution by decreasing neuronal firing, thereby blocking CRH-release into portal circulation (de Kloet *et al.*, 1998a), specifically by inhibiting acetylcholine and serotonin-induced CRH secretion (Hillhouse and Milton, 1989). This stems the liberation of ACTH from the anterior pituitary, causing an interruption in the HPA cascade and thus resulting in stress resolution.

GABA also exerts a regulatory effect on the main peripheral effector organ of the psychological stress response namely the adrenal gland. Studies performed by Kataoka *et al.* (1984) showed that cultures of adrenomedullary chromaffin cells synthesize, store, release

and activate GABA. The GABA receptor complexes located on these cells are functionally coupled to nicotinic receptors and are involved in the modulation of cholinergic-induced catecholamine release. Aside from catecholamine release, activation of nicotinic receptors also stimulates the release of GABA from chromaffin cells which is speculated to in turn reduce nicotine-elicited catecholamine secretion by lowering the trans-synaptic evoked increase in excitability in adrenal medullary cells. This is accomplished by inhibition of adenylyl cyclase activity, the opening of G-protein-activated K^+ channels and inhibition of voltage-gated Ca^{2+} currents (Bowery *et al.*, 2002, Metzeler *et al.*, 2004). Intracellular Ca^{2+} levels are known to play a vital part in the regulation of adrenocortical function (Foster *et al.*, 1997). Recent studies also demonstrate the presence of GABA receptors on steroid-producing adrenocortical cells, suggesting both a paracrine and autocrine function of GABA in modulating steroidogenesis in rat cortical cells (Chessler *et al.*, 2002, Gamel-Didelon *et al.*, 2003, Metzeler *et al.*, 2004).

2.3.5 Brain-Derived Neurotrophic Factor

Acute as well as prolonged exposure to psychological stress and the primary stress hormone, corticosterone, is reported to decrease the expression of brain derived neurotrophic factor (BDNF) in the CA1 region of the hippocampus (Schaaf *et al.*, 1998). BDNF is a growth factor of the neurotrophin family which is secreted in the central and peripheral nervous system as well as in the kidneys and testis (Binder and Scharfman, 2004). Even though the majority of central neurogenesis takes place prenatally, certain areas of the brain maintain the capacity to grow new neurons from neural progenitor cells. This process is modulated by neurotrophins, especially BDNF, which supports the survival of living neurons as well as spurring the proliferation and differentiation of new neurons and synapses (Acheson *et al.*, 1995, Huang *et al.*, 2005). These functions are achieved by the binding of BDNF to at least

two types of receptors on the surface of BDNF-responsive cells in target tissues nl., TrkB (pronounced “Track B”), a tyrosine receptor kinase, is capable of phosphorylating intracellular tyrosine molecules, activating cell signaling, and p75, a low-affinity nerve growth factor receptor (LNGFR) (Patapoutian and Reichardt, 2001). In the brain, BDNF is most active in the prefrontal cortex and the hippocampus, two central areas prominently activated by psychological stress. The down-regulation of BDNF by adrenocortical corticosterone secretion after exposure to chronic immobilisation may explain the occurrence of hippocampal atrophy (Schaaf *et al.*, 1998). *In vivo* studies have also shown hippocampal atrophy in rats bred heterozygously for BDNF (in order to reduce the expression of BDNF), while BDNF knockout mice had severe repercussions including cerebellar abnormalities such as increased sympathetic innervations, and postnatal lethality (Blake *et al.*, 2003). Vollmayr *et al.* (2000) demonstrated that rats exposed to either single or repeated exposures to immobilisation showed markedly lower BDNF mRNA levels in the hippocampal formation. The apparent stress-induced atrophy as well as the decreases in circulating BDNF levels suggests that BDNF may be a noteworthy marker of the psychological stress response and its effects, and that it could be exploited as a predictor of prognosis in acute as well as chronic stress related diseases.

2.3.6 IL-6 and other pro-inflammatory cytokines

In the 30 years since work in the field of psychoneuroimmunology began, studies have convincingly established that stressful experiences alter features of the immune response (Segerstrom and Miller, 2004). It is well known that a variety of stress-related ailments, such as coronary heart disease, have a strong inflammatory component. Certain immune inputs, mediated through cytokines, also regulate the secretion of corticosterone. The most prominent cytokines released in response to psychological stress are IL-1 β , IL-6 and TNF- α

which are secreted in concert by immune cells located within the inner adrenal cortex as well as adrenocortical cells themselves (Gonzalez-Hernandez *et al.*, 1996, Path *et al.*, 1997). Despite the presence of high levels of locally produced glucocorticoids, these above mentioned cells have the ability to maintain synthesis and secretion of pro-inflammatory agents. This phenomenon can most likely be explained by the presence of migration inhibitory factor, which overrides the anti-inflammatory effects of the glucocorticoids (Bacher *et al.*, 1997). Cytokines exert stimulatory and/or inhibitory effects on adrenal function and their expression vary between species. In rats, cytokines originating from the medulla are predominantly illustrated in the cortical zona glomerulosa where it stimulates corticosterone producing cells, while in humans the main site of production is the inner zona reticularis (Judd, 1998).

From the literature consulted, IL-6 seems to exert both pro- and anti-inflammatory effects. The primary functions of this cytokine are pro-inflammatory, for example stimulation of T cell proliferation, and prompting secretion of other pro-inflammatory cytokines and acute phase proteins (Mendel *et al.*, 1998, Marby *et al.*, 2001, Steptoe *et al.*, 2001, Black and Garbutt, 2002, Pedersen and Fischer, 2007, Filiopoulos and Vlassopoulos, 2009). However, IL-6 is also a potent activator of the HPA-axis by neuronal stimulation of the hypothalamic PVN and pituitary to release of CRH and ACTH respectively, leading to the release of glucocorticoids, a very powerful anti-inflammatory hormone (Lyson and McCann, 1991, Spinedi *et al.*, 1992, Jankord *et al.*, 2007). IL-6 has also been proven to inhibit cortisol binding globulin synthesis, thereby increasing the bioavailability of cortisol (Bartalena *et al.*, 1993). Lastly, IL-6 performs an indirect anti-inflammatory function by down-regulating stress-induced TNF- α through a negative feedback loop (Aderka *et al.*, 1989, Schindler *et al.*, 1990, Nukina *et al.*, 1998b).

In vivo studies reported a positive correlation between increases in steroidogenic-stimulators such as ACTH and IL-6 in circulation (Lyson and McCann, 1991, Matta *et al.*, 1992). However, it is not yet known whether or not this correlation follows through to tissue level. Cytokines can influence adrenocortical cells directly by modulating cell growth and differentiation, as well as influencing steroidogenesis; these results are supported by the discovery of IL-6 receptor expression on steroid-producing cells in adrenal glands (Path *et al.*, 1996).

Suggestions have been made that adrenal IL-6 may act in a paracrine fashion to stimulate adrenal secretion of glucocorticoids (Turnbull and Rivier, 1995, Path *et al.*, 1996, Franchimont *et al.*, 2000a, John and Buckingham, 2003). It is known that the adrenal gland is the biggest source of IL-6 in response to psychological stress (Zhou *et al.*, 1993), the origin of which could likely be attributed to splanchnic nerves penetrating the adrenal capsule. It appears that IL-6, along with catecholamines originating from adrenomedullary chromaffin cells, in large amounts stimulate steroidogenesis in a direct manner and probably is one of the primary activators of the adrenal cortex by the sympatho-adrenal medullary system (Bornstein and Chrousos, 1999). It is therefore not surprising considering the neuronal origin of IL-6 that *in vivo* studies using intracerebroventricular injections of GABA_AR and GABA_BR agonists showed decreased restraint stress-induced plasma IL-6 levels while injection of GABA_AR and GABA_BR antagonists resulted in higher basal and stress-induced plasma IL-6 levels (Song *et al.*, 1998). IL-6 therefore constitutes a pivotal component, without which the proper function of the sympatho-adrenal medullary system could not be achieved.

2.4 Potential indigenous anti-anxiety agents

Sutherlandia frutescens (more commonly known as cancer bush) has been used for its medicinal properties for a long time in Southern African countries like South Africa, Lesotho, Namibia and Botswana. It has been used as traditional medicine by a wide variety of cultural groups in these areas including the Zulu, Xhosa, Khoi-San and Cape Dutch. Its uses also vary from the treatment of internal cancers, stomach ailments and diabetes to stress and anxiety. The native words for this plant, including the Sotho name *motlepele*, meaning “bringing back the heart”, or *insiswa*, an ancient Zulu word meaning “he who dispels darkness”, clearly describes a function of this plant in relieving anxiety or depression. The *Sutherlandia* genus is extremely variable with respect to its chemical, genetic and geographic properties and can be subdivided into three subspecies and several regional forms. Some biochemical components of *Sutherlandia frutescens*, subspecies *microphila*, or *S. frutescens*, have been identified by chemical analysis. These include substances known to have anti-viral and anti-cancer properties, like L-canavanine, and others that have mood-elevating properties, such as GABA (van Wyk and Albrecht, 2008). Another component called pinitol has anti-inflammatory as well as insulin-like properties (Brummerhoff, 1969, Viljoen, 1969, Moshe, 1998, Levy *et al.*, 1999). Previous studies done by our group demonstrated that rats receiving chronic daily treatment with *S. frutescens* warm water extract showed significant attenuation of the corticosterone response in when exposed to chronic intermittent restraint stress (Smith and Myburgh, 2004). A complimentary study postulated that the underlying mechanism may involve *S. frutescens*-induced suppression of the cytochrome P450, a catalyst of the corticosterone biosynthetic pathway (Prevo *et al.*, 2004). However, this cannot account for the acute mood-altering effects reported. The exact mechanisms of this plant’s anti-stress action have thus not been fully elucidated and require further investigation. These mechanisms may involve pathways situated in the hippocampus and sympathetic nervous

system. Anecdotally, *S. frutescens* results in a slight “docile” response, not unlike that ascribed to GABA (Mao *et al.*, 1978). Therefore, it is possible for example, that *S. frutescens*-derived GABA down-regulates stress perception inhibiting stress-related sympathetic signaling. These theories are supported by reports of anti-convulsant properties of *S. frutescens* that is thought to be mediated by either acting like GABA or by indirectly enhancing GABAergic neurotransmission (Ojewole, 2008). There may thus also be truth in the claims that *S. frutescens* could improve quality of life in cancer and HIV patients, since both disease states are associated with chronically elevated glucocorticoid secretion. *S. frutescens* has been described as an adaptogen, increasing the body’s ability to adapt to and resist stress and disease through a process called allostasis (maintenance of homeostasis through multiple integrative adaptive processes) (McEwen and Seeman, 1999, Goldstein and McEwen, 2002). *S. frutescens* therefore constitutes a natural complimentary medicine that is worthwhile investigating as a tool against stress, especially in developing countries where access to conventional pharmaceuticals is limited.

2.5 Summary

Despite popular opinion that glucocorticoid secretion in response to stress is primarily mediated by the HPA axis, a growing body of evidence in modern literature suggests that adrenocortical function is regulated by a complex interaction between the HPA axis and the sympatho-adrenal medullary pathway. Changes in corticosterone levels do not always mirror changes in ACTH levels and support the presence of extra-pituitary mechanisms of adrenal regulation (Bornstein and Chrousos, 1999). Even though the exact ratio of control exercised by the respectable pathways is as yet unknown, from an evolutionary point of view, the sympatho-adrenal medullary system most likely constitutes the ancestral pathway. It is therefore not unlikely that glucocorticoid secretion, at least in the acute response to stress,

could be primarily under the control of the sympatho-adrenal medullary system (fig. 2.4). Indicators of psychological stress such as central GABA levels and circulating corticosterone levels, as well as major role players in the two pathways (ACTH and IL-6 for the HPA-axis and sympatho-adrenal medullary system respectively) were identified and could be used to establish an accurate representation of the internal milieu as well as the pathways at work. *S. frutescens* is also speculated to convey its anxiolytic effects through the alteration of stress perception by the induction of inhibitory GABAergic transmission in the hippocampus, the major source of inhibitory input to the peripheral limbs of the psychological stress response.

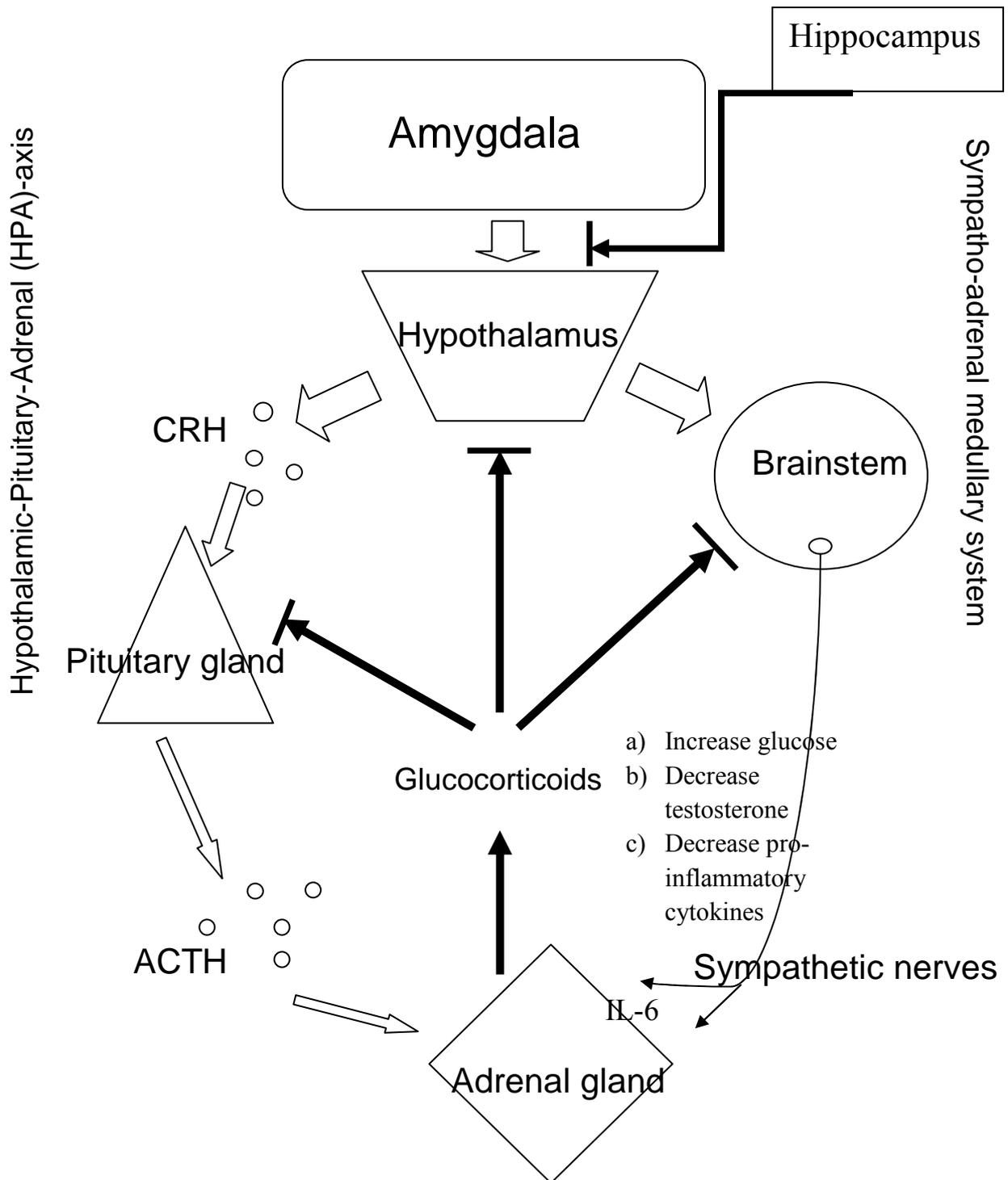


Figure 2.4. Schematic representation of the pathways involved in the stress signal transduction.

2.6 Hypothesis & aims

After a thorough review of the literature, we hypothesised the following in the context of acute psychological stress:

- a. The sympatho-adrenal medullary system constitutes the primary and the HPA-axis the secondary response pathway.
- b. Since *S. frutescens* contain GABA and has anti-inflammatory properties, it may exert its anxiolytic effect *via* suppression of the sympatho-adrenal medullary system.

We aimed to test our hypothesis by:

1. Inducing an acute mild psychological stress response by means of restraint in male Wistar rats
2. Blocking IL-6 – a major role player in the sympatho-adrenal medullary system - by administration of an anti-IL-6 antibody to probe the importance of this pathway
3. Investigating the effects of *in vivo*, acutely administered *S. frutescens* on psychological stress by assessment of alterations to central and peripheral limbs of both the HPA-axis and sympatho-adrenal medullary mediated psychological stress responses.

To address these aims, I conducted two separate experiments, both using an established rodent model for acute mild psychological stress. These experiments will be described in the next two chapters, in terms of methodology, results and data interpretation. In the final chapter, conclusions will be drawn and directions given for further study.

Chapter 3 : The role of IL-6 in the acute response to psychological stress

3.1 Introduction

With regard to psychological stress, the sympatho-adrenal medullary system has long been thought to only be involved in catecholamine secretion from the adrenomedullary chromaffin cells. Evidence of synaptic activity amongst adrenocortical cells has spurred a unified approach to adrenal regulation, especially in context of stress-induced activation by sympathetic neuronal inputs, since it likely constitutes the evolutionary ancestral pathway. We therefore attempt to fully elucidate the importance of the sympatho-adrenal medullary system in the acute response to psychological stress by blocking one of its key components, IL-6.

3.2 Methodology

3.2.1 Experimental animals:

Forty male Wistar rats (weighing 372 ± 66 g) were bred in a local small animal unit and housed in standard rat cages. Rats were fed standard rat chow and tap water *ad libitum*, while the ambient temperature was kept constant at 21°C. Rats were subjected to a 12-hour light/dark cycle (lights on at 7 am). Sufficient ventilation was provided at a rate of 10 room changes per hour. For four-five weeks prior to initiation of experimental protocols all rats were purposefully handled to accustom them to the investigators, thereby minimizing confounding effects of acute experimental stress-variables not related to the intervention treatments.

3.2.2 Ethical considerations

All protocols used were approved by the Animal Ethics Committee of Sub-Committee B of Stellenbosch University (ref # 20040218CS), and interventions carried out in accordance with ethical guidelines of the South African Medical Research Council.

Detailed intervention protocol for the first experiment performed in this study is described below:

3.2.3 Protocol

Rats were divided into eight weight-matched groups of five animals per cage, and cages randomly assigned to the following 4 experimental groups (i.e. n=10 per group): Control Placebo (CP), Control Antibody (CA), Stress Placebo (SP) and Stress Antibody (SA).

The timeline for all interventions are presented in Fig 3.1, followed by a more detailed description of each intervention.

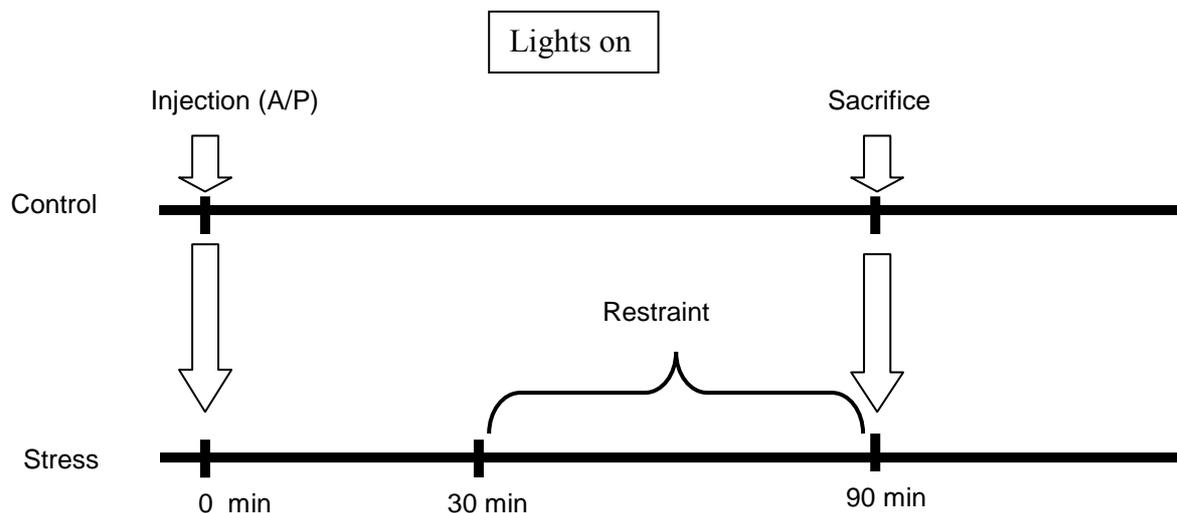


Figure 3.1. Schematic representation of experiment 1. Abbreviations: A, antibody; P, placebo; min, minutes.

3.2.3.1 Interventions

IL-6 Antibody preparation:

To prepare the IL-6 antibody, 100 µg lyophilised goat-derived antibody to rat IL-6 (AF506, R&D Systems, Germany) was dissolved in 1 ml of sterile saline according to manufacturer's instructions. This 100 µg/ml stock solution was subsequently diluted 1:50 in sterile saline to yield a final concentration of 2 µg/ml. One injection per rat were administered i.p. at a dosage of 2 µg/kg body weight to groups CA and SA, while sterile saline was administered (1ml/kg body weight) to placebo groups (CP and SP). Rats were allowed 30 minutes to ensure thorough absorption of the antibody before commencing with stress exposure.

Stress model:

Stress group rats (SP & SA) were subjected to restraint for a period of 60 minutes in purpose-made individual Perspex cages (dimensions 7 cm x 8 cm x 15 cm), and sacrificed within 5 min after removal from these cages. Placebo rats were returned to their housing cages for the time period between injection and sacrifice. Sacrifice time points for control rats were chosen to correspond to that of stressed rats, so that all rats were sacrificed at a time point 90 minutes after injection of either placebo or the IL-6 antibody.

3.2.3.2 Sacrifice & Sample collection

Animals were sacrificed by pentobarbitone sodium overdose (200 mg/kg i.p.). To exclude influences of circadian rhythms on circulating hormone concentrations, all animals were sacrificed between 8 and 10 am. After loss of consciousness, but before cardiac arrest, blood was collected from the heart by means of left ventricular puncture using a sterile 20 gauge, 3.75 cm long needle and a 5 ml sterile syringe, and transferred into lithium heparin tubes (Vacutainer, BD Systems, Plymouth, UK). Tubes were kept on ice and centrifuged at 3000

rpm for 10 minutes at 4°C within an hour of collection. Plasma was aliquotted and stored at -80°C until subsequent batch analysis of cytokine and corticosterone concentrations. Adrenal and pituitary glands were dissected out, cleaned of visible fat and connective tissue and weighed on an electronic balance accurate to the nearest milligram (Shimadu, type AW220, Phillipines Manufacturing Inc. Japan). Tissue was then fixed in 10% formal-saline for 3 days, after which it was processed into paraffin wax blocks using standard histological procedures. Between consecutive sacrifices all blood and animal waste were removed and the working area disinfected to prevent an acute stress response as a result of the odours of previously sacrificed rats.

3.2.3.3 Sample analysis

Wax embedded tissue samples were used to prepare 5 µm sections using a rotary microtome (Reichert Jung, Heidelberg, Austria). These sections underwent conventional de-paraffinisation and rehydration processing prior to initiation of staining protocol. Routine H&E staining as employed for assessment of histological changes, while more advanced immunohistochemical techniques were used (see appendix C for detailed description of protocol) to confirm the availability of IL-6 antibody (raised in goat) at tissue level using a Texas Red conjugated secondary anti-goat antibody (sc-2783, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Haematoxylin and eosin (H&E) stains were also prepared using standard histological techniques.

A commercially available enzyme-linked immune-sorbent assay (ELISA)-based bead kit system (Bio-Plex, BioRad Laboratories Inc., Parklands, South Africa; refer to Appendix A for details) was used for the determination of plasma IL-1 β , IL-6, TNF- α concentrations, and enzyme-immune-assay (EIA) kits (AC-14F1, Immunodiagnostic Systems Ltd., Frankfurt,

Germany) for corticosterone concentrations in duplicate. Briefly, the Bio-Plex assay employs multiplexing technology that uses multiple colour-coded bead sets (three sets for this particular experiment), each conjugated with a specific primary antibody (in this case IL-6, IL-1 β or TNF- α) *via* a covalent bond. These antibody-coupled beads are incubated with samples and standard solutions to bind to the target molecules, after which unbound proteins are washed away. A biotinylated detection antibody specific for a different epitope on the cytokine (i.e. another primary antibody) is added to the reaction solution, resulting in formation of a sandwich of antibodies around the cytokine. For detection of fluorescence, phycoerythrin labeled streptavidin (streptavidin-PE) is added and binds to the biotinylated primary antibodies. Finally, the content of each well is aspirated by an automated Bio-Plex reader, which identifies the parameter (cytokine) according to the colour of the bead bound to it, and concentration by fluorescence intensity (a detailed protocol provided in Appendix A).

The principle of the EIA assay is based on the competitive binding between the endogenous molecule of interest and a horseradish peroxidase conjugate. These two competitively bind to antibody-coated microtiter wells during incubation. After incubation the unbound peroxidase conjugate is washed off. The addition of a substrate solution spurs colour development of the conjugate and the intensity of the colour development (assessed by colorimetry using a 96-well plate reader) is inversely proportional to the concentration of the molecule of interest. (Detailed protocol provided in Appendix B).

3.2.3 Statistical Analysis

Values for IL-6 were very low across board, with detectable concentrations in less than 50 % of samples. Therefore, an arbitrary value of zero was assigned for non-detectable samples, in order to facilitate statistical comparison between groups. Data for IL-6 were not normally distributed – therefore group differences were assessed using Kruskal-Wallis ANOVA by ranks. Differences

between groups for all other parameters were assessed using Factorial ANOVA and Fisher *post hoc* tests (Statistica v.8, StatSoft Software). $P < 0.05$ was set as level of significance. All results are reported as means and standard error of means (SEM).

3.3 Results

Immunohistochemical analysis of the pituitary and adrenal glands confirmed that i.p. injection of the IL-6 antibody did indeed result in the anti-IL-6-antibody becoming bioavailable at tissue level, as indicated by staining for the antibody in different target tissues (Fig 3.2). This data further suggest that at the point of sacrifice and sample collection, the anti-IL-6 antibody had indeed bound to IL-6 (which would have “sequestered” the antibody in the particular tissue).

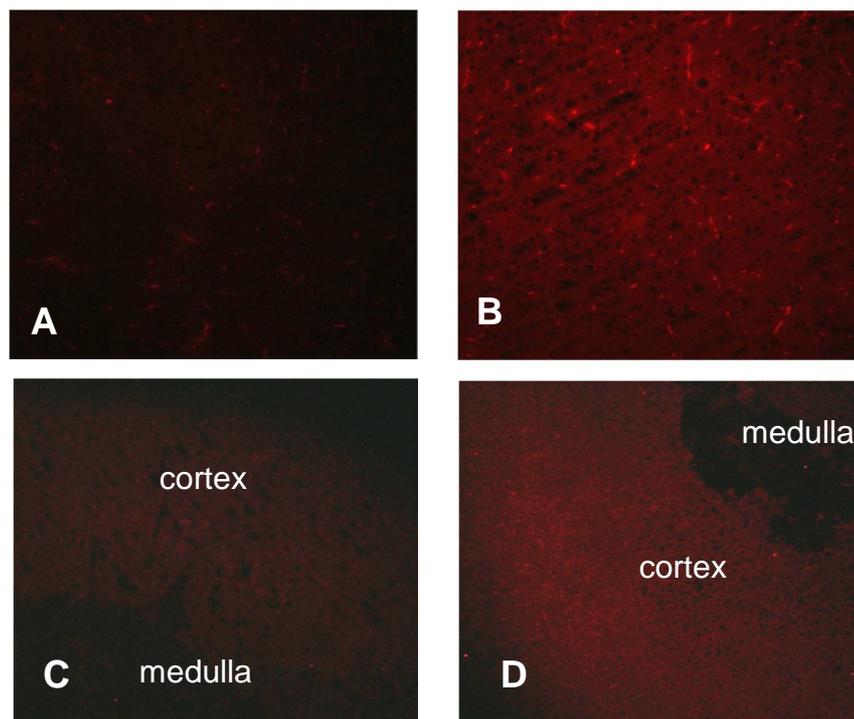


Figure 3.2. Figures are representative samples ($n = 4$) of posterior pituitary tissue (magnification 200x) in placebo rats (A) and those injected with anti-IL-6 antibody (B), and adrenal tissue (magnification 100x) of placebo (C) and antibody-treated rats (D).

Stress had a significant main effect on plasma corticosterone levels ($P < 0.0001$), with stress resulting in a more than 5-fold increase in corticosterone levels ($P < 0.0001$; Fig 3.3), a response attenuated when IL-6 was blocked (ANOVA interaction effect of stress and antibody treatment, $P < 0.0001$).

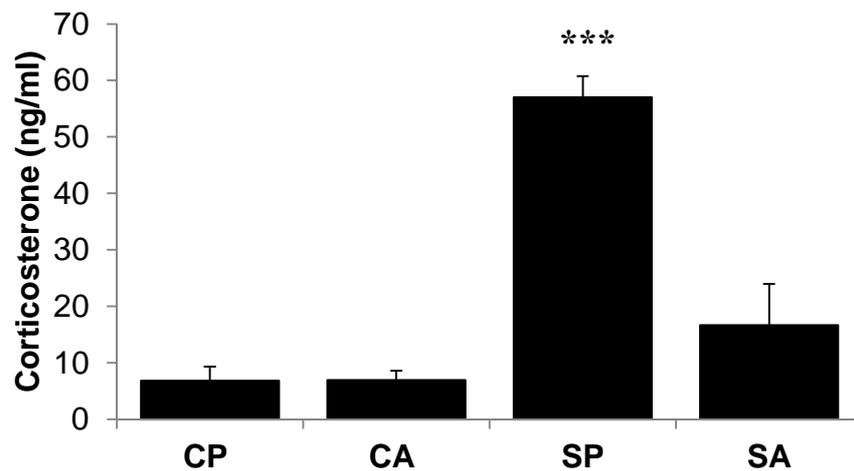
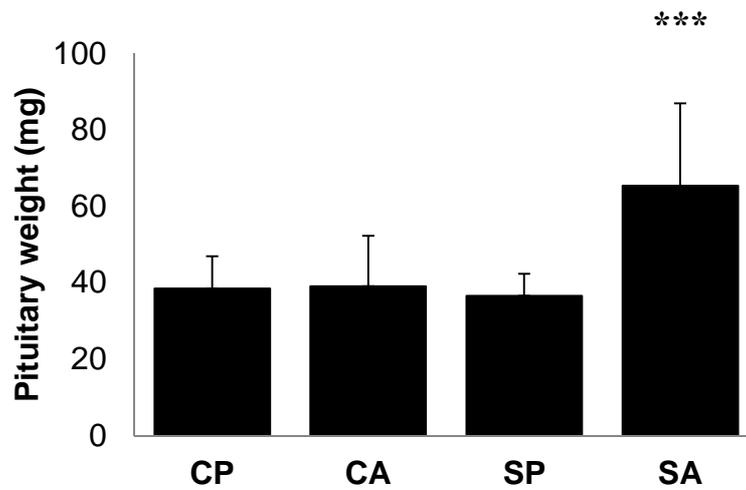


Figure 3.3. Effect of acute restraint stress in the presence or absence of intact IL-6 on plasma corticosterone levels, $n = 10$. Abbreviations: CP, control placebo, CA, control antibody-treated, SP, stress placebo, SA, stress antibody-treated.

***: $P < 0.0001$, different from all other groups

Significant effects of stress and/or the blocking of IL-6 were observed for both pituitary and adrenal tissue masses. In rats exposed to both stress and IL-6 antibody (SA), pituitary mass was significantly greater when compared to all other groups ($P < 0.0001$; Fig 3.4a), while adrenal mass was only increased in stressed rats with intact IL-6 (SP) ($P < 0.0001$). Of interest is the fact that in this group, the increase in adrenal mass was accounted for by increased gland size on the left side only (Fig 3.4b). Average right adrenal glands showed no significant differences between groups and were measured as 34.7 ± 1.5 mg for CP, 35.5 ± 1.3 mg for CA, 37.2 ± 1.7 mg for SP and 36.6 ± 1.8 mg for SA.

(a)



(b)

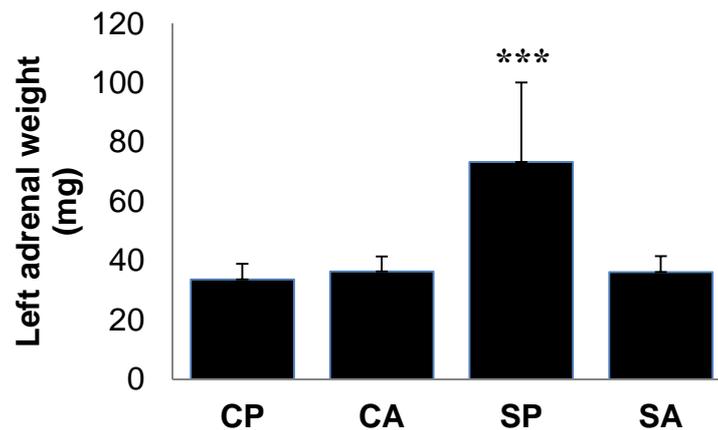
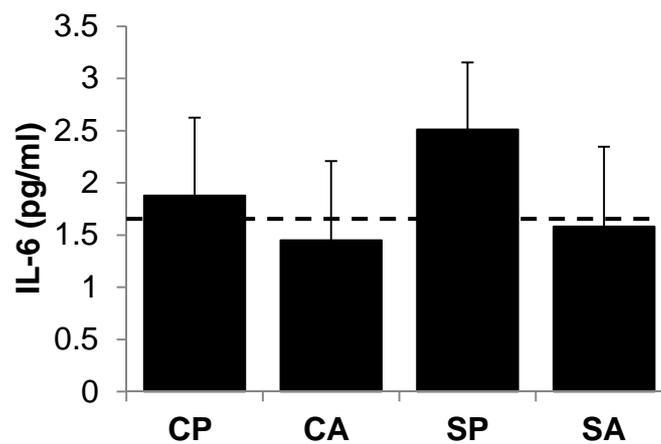


Figure 3.4. Average mass of pituitary gland (a), and left adrenal gland (b). $n = 10$. Abbreviations: CP, control placebo, CA, control antibody-treated, SP, stress placebo, SA, stress antibody-treated
***: $P < 0.0001$, different from all other groups.

Neither stress, nor anti-IL-6-antibody administration significantly affected circulating IL-6 concentrations (Fig 3.5a). Plasma IL-1 β concentration exhibited an ANOVA main effect of antibody treatment ($P < 0.01$) that was independent of stress, with *post hoc* analysis indicating significantly higher values for CP when compared to both antibody-treated groups (CA and SA; Fig 3.5b). TNF- α levels exhibited an ANOVA main effect of antibody treatment

($P < 0.01$), as well as a significant interaction effect of stress and antibody treatment ($P < 0.05$; Fig 3.5c). *Post hoc* analysis indicated lower TNF- α values in antibody-treated rats, an effect attenuated in response to stress. (Lower detection limits of the cytokine assay kits used were 0.2, 1.7 and 0.2 pg/ml for IL-1 β , IL-6 and TNF- α respectively.)

(a)



(b)

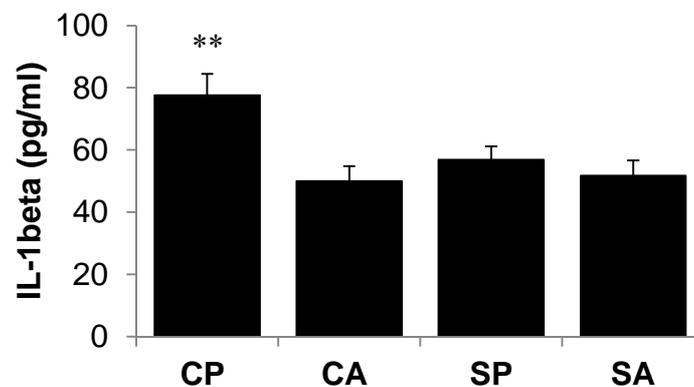


Figure 3.5. Effect of acute restraint stress in the presence or absence of intact IL-6 on inflammatory cytokines: (a) IL-6 and (b) IL-1 β , $n = 10$. Abbreviations: CP, control placebo, CA, control antibody-treated, SP, stress placebo, SA, stress antibody-treated. Broken line represents lower detection limit (1.7 ng/ml) of assay.

** : $P < 0.001$, different from all other groups

(c)

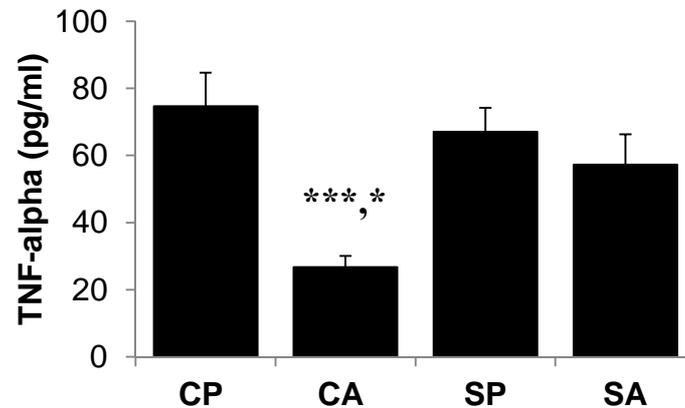


Figure 3.5 (continued). Effect of acute restraint stress in the presence or absence of intact IL-6 on inflammatory cytokine: (c) TNF- α , n = 10. Abbreviations: CP, control placebo, CA, control antibody-treated, SP, stress placebo, SA, stress antibody-treated.

***: P<0.001, different from CP and SP; *: P<0.05, different from SA.

3.4 Discussion

The main findings reported here, as assessed at a single time point after 60 minutes of acute restraint in rats are a) that restraint stress led to a significant increase in plasma corticosterone levels compared to control groups only in the presence of IL-6, b) an increase in left adrenal mass in response to stress which was attenuated in the absence of IL-6, c), an increase in pituitary weight during stress only when IL-6 was blocked and d) decreased IL-1 β and TNF- α concentrations when blocking IL-6 in the absence of stress.

Although average IL-6 concentrations in antibody-treated groups were somewhat lower when compared to their respective controls, these differences did not reach statistical significance. All IL-6 levels were very low, which in combination with high variability between animals, may have obscured both an effect of stress and the effect of blocking IL-6 using the antibody. Another likely possibility is that the transient increase in IL-6 release, known to occur *via* adrenaline stimulation in response to stress (DeRijk *et al.*, 1994), was never detectable in circulation. In a study performed by Uchiyama *et al.* (2008), it was found that in models of

collagen-induced arthritis elevations in blood IL-6 was only detectable after blocking of IL-6 receptor (IL-6R). They concluded that the elevations were only seen due to the blocking of the IL-6-clearance effect brought on by IL-6R (Uchiyama *et al.*, 2008). It is therefore reasonable to argue that plasma IL-6 levels do not accurately reflect the events at tissue level, considering it has its primary function at tissue level where it is secreted, resulting in only a fraction of secreted IL-6 reaching the circulation. Despite this, since we were able to illustrate the presence of IL-6 antibody at tissue level, and measured circulating IL-6 levels below the assay detection threshold in 70% of antibody-treated animals, we are confident that we did in fact block IL-6 to an extent sufficient to evaluate its role in the stress response.

In previous studies by our group, repeated restraint stress induced up to ten-fold increases in serum corticosterone levels (Smith and Myburgh, 2004, Smith *et al.*, 2007) Our findings of increased corticosterone is in agreement with this, as well as similar reports by other research groups (Pitman *et al.*, 1988, Dronjak *et al.*, 2004), in both acute and chronic models. The attenuated corticosterone response to stress when IL-6 was blocked (SA) supports an earlier report of direct stimulation of adrenal cortical cells by IL-6 to secrete corticosterone in an *in vitro* model (Franchimont *et al.*, 2000a). Although neuronal-derived IL-6 has been shown to play an integral part in corticosterone secretion in *in vivo* models of repeated psychological stress (Smith *et al.*, 2007), this is the first study showing this role of IL-6 in an *in vivo* model of acute psychological stress.

The finding that adrenal mass increased in response to stress only when IL-6 was intact, further points to a direct stimulatory effect of IL-6 at adrenal level. These results are in agreement with the literature (Turnbull and Rivier, 1995, Path *et al.*, 1996, Franchimont *et al.*, 2000a, John and Buckingham, 2003). Of special interest, is the finding that mainly left adrenal mass increased in stressed animals with intact IL-6. From the literature, we know that asymmetry exists in the intensity of splanchnic innervation of the adrenals, with the left

adrenal receiving more cerebral neural inputs than the right adrenal (Toth *et al.*, 2008a, Toth *et al.*, 2008b). The functional significance of the asymmetry in the control of adrenal function is not yet known. An influencing factor of the left adrenal hypertrophy could be the light/dark cycle used. The contribution of this factor will be discussed in section 4.4. Our model seems to suggest that the central sympathetic signal travelling through the splanchnic nerves to the adrenal medulla stimulates the adrenomedullary chromaffin cells to release IL-6, which act in a auto-/paracrine fashion to stimulate glucocorticoid secretion from the adrenocortical cells. The primary signal transduction in response to psychological stress is therefore transmitted *via* the sympatho-adrenomedullary pathway rather than systemically *via* the circulating stress-associated hormones, a.k.a. the HPA axis.

H&E analysis of adrenal glands supports this result by showing marked increases in the number of activated adrenocortical cells of the zona fasciculata in left adrenals (Fig. 3.6A) compared to right adrenals (Fig. 3.6B) in SP rats. It is therefore certain that hyperplasia, and possibly hypertrophy, can account for increases in adrenal mass. Decreased stores of intercellular cholesterol droplets or liposomes were also observed in left adrenal glands, indicating higher substrate utilization and therefore increased cellular activity. Literature indicates that the above mentioned characteristics are indicative of higher glucocorticoid production in adrenocortical cells (von Euler, 1967).

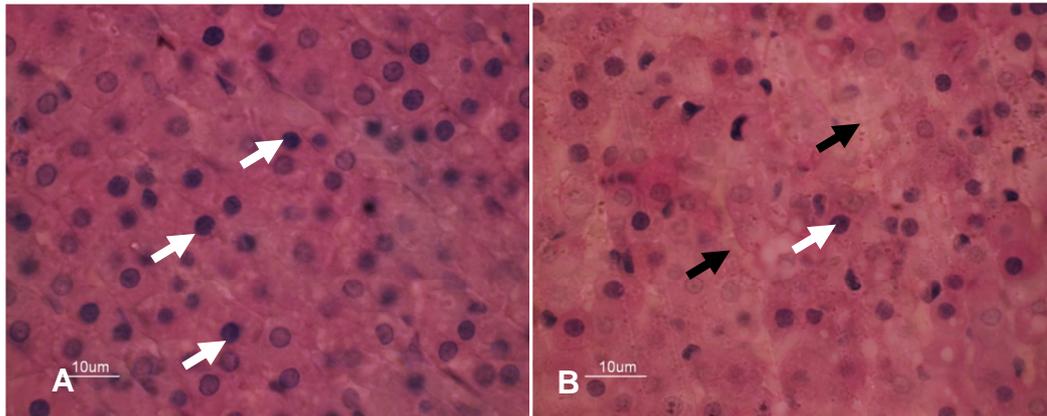


Figure 3.6. Effect of acute restraint stress on left (A) vs. right (B) adrenocortical cell activation with white arrows indicating activated cell nuclei and black arrows indicating liposomes, n = 4.

Pituitary gland mass was not influenced by either blocking IL-6 in absence of stress, or by stress alone. However, in contrast to the results obtained in the adrenals, only when blocking IL-6, did restraint stress result in a marked increase in pituitary mass. This suggests a stimulatory effect of the gland in the absence of IL-6. There is more than one possible explanation for this result. Firstly, IL-6 may produce direct negative feedback to the pituitary to limit HPA-associated corticosterone release. This is however unlikely, since IL-6 is known to stimulate the pituitary gland to secrete ACTH by increasing phosphorylation of signal transducer and activator of transcription (STAT)-3 in pituitary corticotrophs (Jankord *et al.*, 2007). Secondly, it is possible that the lower corticosterone concentration seen in SA compared to SP may have provided too little negative feedback to the pituitary, resulting in continued activity of the gland, thus suggesting a more indirect central action for IL-6. However, the fact that pituitary mass did not change at all in SP, where corticosterone release increased almost 6-fold, argues against a major role for the pituitary in this response. A third, more likely, alternative is that while sympathetically-induced corticosterone release was possible when IL-6 was intact (SP), in SA this pathway was largely incapacitated, and required the endocrine support facilitated *via* the traditional HPA-axis to accomplish a

glucocorticoid response, resulting in the increased stimulation of the pituitary and therefore increased pituitary mass.

Stress alone had no significant effect on TNF- α release, a result supported by the literature, since TNF- α release has previously been shown to be unresponsive to both chronic and more acute psychological stressors (Goebel *et al.*, 2000, Smith and Myburgh, 2004). The decrease plasma IL-1 β concentrations seen in SP compared to CP was probably due to an anti-inflammatory effect of stress-induced increase in corticosterone.

The blocking of IL-6 resulted in decreased IL-1 β and TNF- α levels in control animals. This is not in agreement with the effect reported for IL-6 in the stress literature, where IL-6 is known to inhibit IL-1 β production in models of sepsis (Aderka *et al.*, 1989, Arbo *et al.*, 1990, Schindler *et al.*, 1990, Barton and Jackson, 1993). However, the current result obtained from control animals is in agreement with previous work by our group in a chronic restraint stress model, which also suggested a role for IL-6 in maintaining basal IL-1 β levels (Smith *et al.*, 2007). It is also further supported by an earlier report that *in vitro* IL-6 administration increased IL-1 β mRNA levels in leukocytes in the absence of an added stressor (Panzer *et al.*, 1993). IL-1 β has also been implicated, along with IL-6, to constitute an integral part of sympathetic neurotransmission (Xia *et al.*, 1999). Therefore, the decreased ability to maintain plasma IL-1 β levels in the absence of IL-6 supports our notion for decreased sympathetic nervous excitation. With regard to TNF- α , IL-6 seemed to also have a role to ensure its continued release, an effect again only evident in unstressed conditions. This is again in contrast with the literature, which suggests a role for IL-6 in down-regulation of TNF- α (Aderka *et al.*, 1989, Schindler *et al.*, 1990, Nukina *et al.*, 1998a). However, the inhibitory effect of IL-6 on LPS-induced TNF- α secretion was previously shown to be limited to macrophage lineages, while microglial cells were not affected (Di Santo *et al.*, 1997), suggesting that the down-regulatory effect does not occur in a homologous fashion in all cell

types. Furthermore, the above-mentioned reports of IL-6-associated inhibition of TNF- α release were generated from assessments in non-physiological models of stress or pathology such as sepsis, and, as in the case of IL-1 β , not under unstressed conditions. From this literature, it is not possible to conclude what the effect of IL-6 on TNF- α release would be under basal conditions. It is therefore possible that under basal conditions IL-6 could exert an opposite effect on TNF- α release, similar to its reciprocal effect on IL-1 β , as reported here.

Taken together, these results suggest that in the immediate response to acute stress, the stimulatory effect on the adrenal cortex to initiate glucocorticoid release is under direct neural control, facilitated through the sympatho-adrenal medullary system. Whether or not this extrapituitary-adrenocortical stress response is evolutionary exclusive or additive is important in order to understand the biological relevance of such a pathway. The answer to this question seems to be species-dependent, with only mammalian organisms requiring ACTH for normal adrenocortical function. In non-mammalian species, on the other hand, the steroidogenic function of ACTH can be fulfilled by local paracrine mechanisms involving IL-6. (These mechanisms were discussed in section 2.3.6.) Even though rodents in fetal or early postnatal stages of life may maintain adrenocortical steroidogenesis without pituitary ACTH secretion, in adult mammals the loss of pituitary ACTH leads to adrenal atrophy (Bornstein and Chrousos, 1999). Since the HPA-axis is not needed in non-mammalian species, and only becomes essential for survival during later stages of life in mammals, it likely constitutes an evolutionary derived pathway with the sympatho-adrenal medullary system forming the evolutionary ancestral stress pathway. Our results support this theory since pituitary hypertrophy only occurred during the blocking of the sympatho-adrenal medullary system with no changes in plasma corticosterone seen while this system was suppressed (Fig. 3.7).

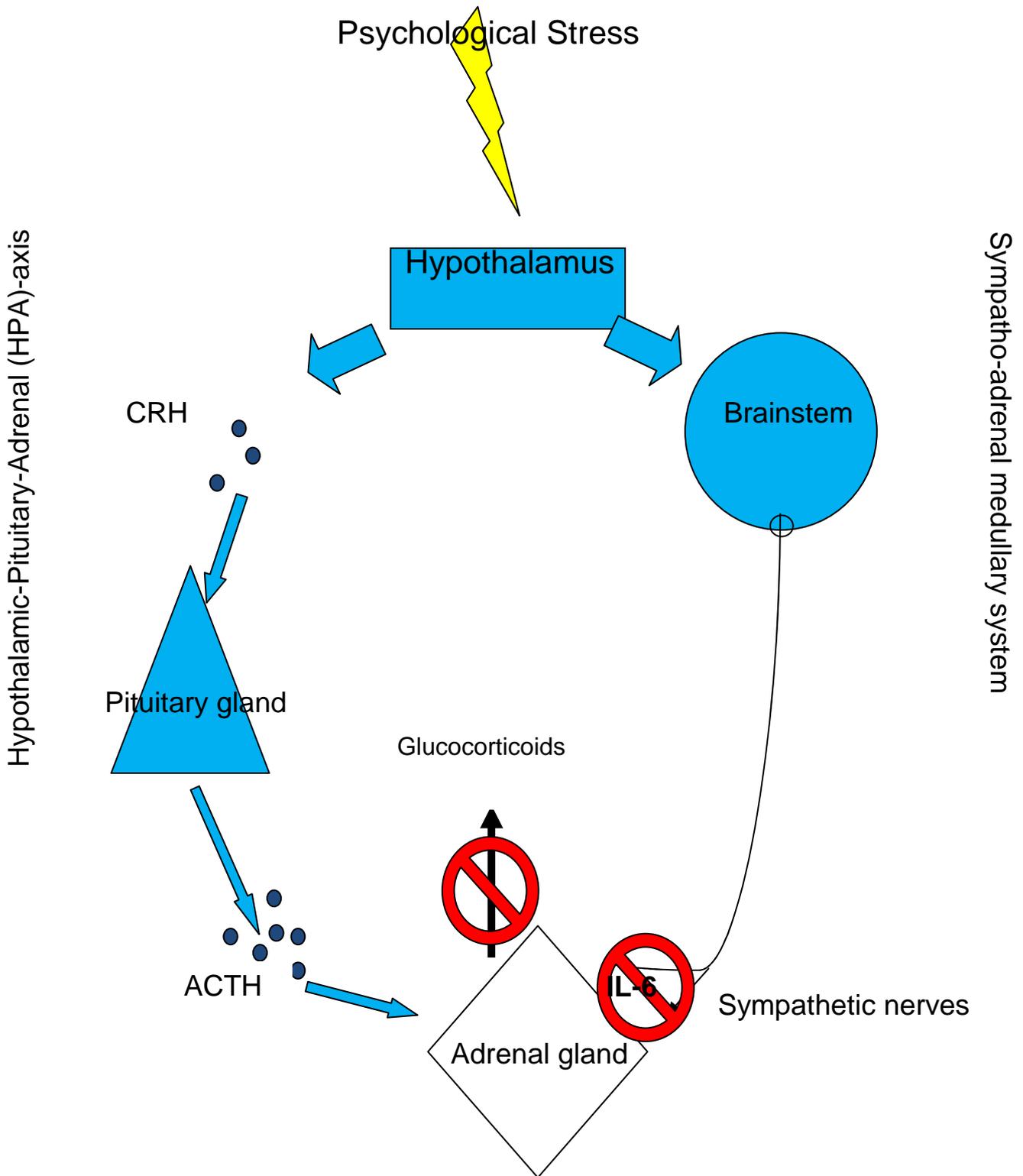


Figure 3.7. Schematic representation of the pathways involved in stress signal transduction after blocking IL-6 with blue indicating the activated pathway.

Chapter 4

Central and peripheral effects of *S. frutescens* in the context of the acute response to psychological stress

4.1. Introduction

Depression, along with other anxiety-related ailments, is increasingly prevalent globally, especially in developing countries such as South Africa (Yusuf *et al.*, 2001). Depression is also known to be highly prevalent precursor to suicide (Heila *et al.*, 1997), which was recently reported to be a major cause of death in South Africa (Stafford *et al.*, 2008). Access to health care is limited, with only 15% of the population covered by private medical aid systems (Eastman, 2005). Traditional healers outnumber conventional western health professionals by at least ten to one (Morris, 2001). *S. frutescens* therefore constitutes a viable and easily obtainable alternative to conventional pharmaceuticals and merits further investigation. Before large scale distribution, the mechanisms involved in the reported anxiolytic effects must be elucidated to exclude possible side-effects not evident from anecdotal evidence or the limited number of scientific studies. Even though the application of *S. frutescens* will likely be chronic we will be using an acute model in order to facilitate assessment of the central effects of *S. frutescens*.

4.2 Methodology

4.2.1 Experimental animals

Wistar rats were bred, fed and housed under similar conditions as the previous experiment with the exception of a reversed light dark cycle (lights off at 7am).

4.2.2 Ethical considerations

All protocols used were approved by the Animal Ethics Committee of Sub-Committee B of Stellenbosch University Council (ref # 2009B02001), and interventions carried out in accordance with ethical guidelines of the South African Medical Research.

Detailed intervention protocol for the second experiment performed is described below:

4.2.3 Protocol

Forty rats were divided into weight-matched groups of five animals per cage, and four experimental groups (n=10 per group) randomly assigned as follows: Control Placebo (CP), Control *S. frutescens* (CSu), Stress Placebo (SP) and Stress *S. frutescens* (SSu). As in chapter 3, the timeline of the intervention protocol is illustrated below (Fig. 4.1), followed by a more detailed description of procedures.

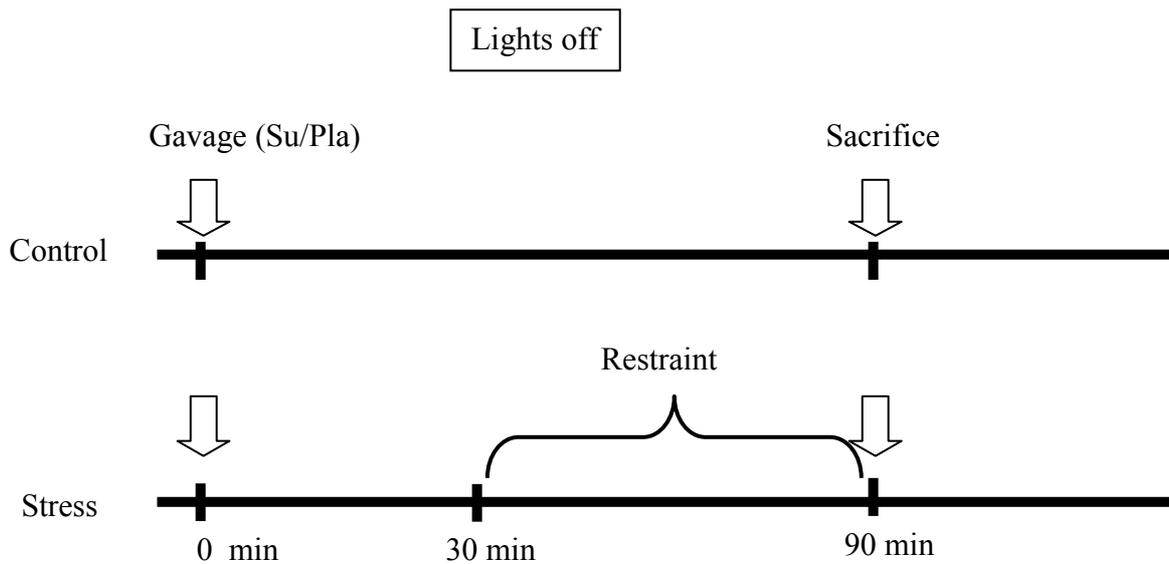


Figure 4.1. Schematic representation of interventions in experiment 2. Abbreviations: Su, *S. frutescens*; Pla, placebo; min, minutes.

4.2.3.1 Interventions

S. frutescens-treatment:

The *S. frutescens* used was harvested in the vicinity of Murraysburg, Western Cape Province, South Africa, and identified as *S. frutescens* subspecies *mycrophilla* by Prof B-E van Wyk from the University of Johannesburg Botany department (Voucher specimen B-E. van Wyk 4126 (UJ)). According to the recommended dosage of commercially available *S. frutescens*, 9 mg per kg body mass per day is the optimal dose in humans. This dose was translated and rounded up to a daily effective dose in rats of 4 mg per kg. Traditionally, *S. frutescens* is administered as an herbal infusion – in keeping with this, we prepared a warm water extract by adding dried *S. frutescens* leaves to boiling water (4mg/ml), allowing infusion with agitation for an hour. To minimise confounding effects of variations in plant material, the *S. frutescens* extract was prepared in one batch and aliquots frozen, for single use at each (staggered protocol) treatment time point. Although each rat was supplemented only once,

entrance into protocol was staggered to allow for synchronized diurnal rhythm resulting in more than one treatment time point. Since the extract is traditionally administered orally, rats were orally gavaged with 1 ml/kg body mass only once, as indicated in Fig. 4.1. The *S. frutescens* extract was prepared in tap water to simulate the historical use thereof and therefore placebo rats were given tap water at a dose of 1ml/kg body weight.

Stress model:

Stress group rats were restrained for a period of 60 min in a purpose-made Perspex cage (dimensions 7 cm x 8 cm x 15 cm) starting 30 min after *S. frutescens* treatment, and sacrificed within 5 min of removal from these cages. Placebo rats were kept under normal housing conditions and in standard cages for this same time period. All rats were thus sacrificed at a time point 90 minutes after gavage with *S. frutescens* extract.

4.2.3.2 Sacrifice & Sample collection

Since this study had a neurophysiology component, we attempted to avoid euthanasia-induced alterations in the central components of stress circuitry by using decapitation as method of sacrifice. Once again, all animals were sacrificed between 8 and 10 am to exclude influences of circadian rhythms on circulating hormone concentrations. Blood was collected from jugular bleeding resulting from decapitation, into heparinised tubes (Vacutainer, BD systems, Plymouth, U.K.). Blood tubes were kept on ice and centrifuged at 3000 rpm for 10 minutes at 4 °C within an hour of collection. Plasma was aliquotted and stored at -80 °C until subsequent batch analysis of testosterone, ACTH and corticosterone concentrations. Rat brains were dissected out and mid-sections frozen in OCT in liquid nitrogen-cooled isopentane. Whole adrenal and pituitary glands were also dissected out, cleaned and weighed on an electronic balance accurate to the nearest milligram. They were then fixed in 10% formal-saline for three days, after which it was processed into paraffin wax blocks using standard histological procedures. Between consecutive sacrifices all blood and animal waste

were removed and the working area disinfected to prevent an acute stress response due to the odours of previously sacrificed rats.

4.2.3.3 Sample analysis

Plasma samples were analyzed using EIA kits according to the manufacturers' instructions for ACTH (Biomerica, Newport Beach, California, USA) corticosterone (Immunodiagnostic-systems, Boldon Business Park, UK), and testosterone (DRG Diagnostics GmbH, Germany) (Appendix B).

Brain tissue samples were prepared into 10 μ m cryosections using a cryostat (CM 1100, Leica Nussloch, Germany) and subjected to immunohistochemical analysis (Appendix C) of GABA_AR α 1 and GR levels. GABA_AR was detected using an anti-human GABA_AR goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) raised against the GABA_AR α 1 subunit of the receptor followed by a Texas Red conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). The GR was detected using a rabbit polyclonal IgG raised against human GR (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) and visualized by using a fluorescent conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA).

Pituitary and adrenal tissue samples were cut into 5 μ m sections using a rotary microtome (Leica RM 2125, Germany) and prepared for immunohistochemical analysis of ACTH expression as well as IL-6 levels in the adrenal tissue (Appendix D). Immunoperoxidase staining of tissue sections was carried out using the avidin-biotin complex (ABC) staining system (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) according to the manufacturers' instructions. Pituitary and adrenal samples were incubated with a monoclonal primary anti-mouse ACTH IgG (Novus Biologicals, Littleton, Colorado, USA). For the detection of IL-6 in adrenal tissue only, a polyclonal anti-mouse IL-6 IgG (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) was used.

4.2.3.4 Image analysis

Two sections per slide were prepared and a negative control stain (PBS control) was performed with both secondary antibodies in order to verify true positive staining. Photos were taken of three fields of view in the hippocampus (CA1 regions) of every section, two sections per slide. One slide per sample was made for four samples in every group. Photos were taken at 40x magnification using a microscope (Nikon ECLIPSE E400; 400x objective used), equipped with a colour digital camera (Nikon DXM1200) and computer software for acquiring digital images (*Simple PCI* version 4.0, Compix Inc., Imaging Systems, USA). All photos were taken using identical filters, exposure times and sensitivity settings. 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: $\text{gray} = (\text{red} + \text{green} + \text{blue}) / 3$. 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 has a subjective component in that the researcher has to adjust image brightness to exclude excessive background staining or increase sensitivity. Nevertheless, this is common practice, and in the current study, all analysis was performed by one researcher (the candidate) only, thereby avoiding variation in the data due to researcher-specific differences in means of analysis. Once a specific profile of settings has been optimized, these settings were used for all images for a particular magnification and parameter.

4.2.4 Statistical analysis

Values obtained for ACTH were very low, with many values lower than the allowed detection limit of kits used, and therefore an arbitrary value of zero was assigned to these values, in order to facilitate statistical comparison between groups. N = 10 were used for the analysis of plasma concentrations of ACTH, corticosterone and testosterone while an n = 4 were used during immunohistochemical analysis of the hippocampal GABA_AR α 1 and GR as well as adrenal ACTH and IL-6 levels. Correlation analysis between circulating corticosterone and hippocampal GR were performed. Differences between groups for all parameters were assessed by Factorial ANOVA and Fischer *post hoc* tests (Statistica v.9, StatSoft Software). P<0.05 was set as level of significance. All results are reported as means and standard errors of means (s.e.m.).

4.3 Results

One-way ANOVA analysis indicated a significant main effect of stress on serum corticosterone levels with increased levels found in both groups exposed to restraint, when compared to their respective controls (P < 0.0001, Fig 4.2). *S. frutescens* did not have a statistically significant effect on corticosterone levels. Lower detection limit for this assay was 1.7 ng/ml.

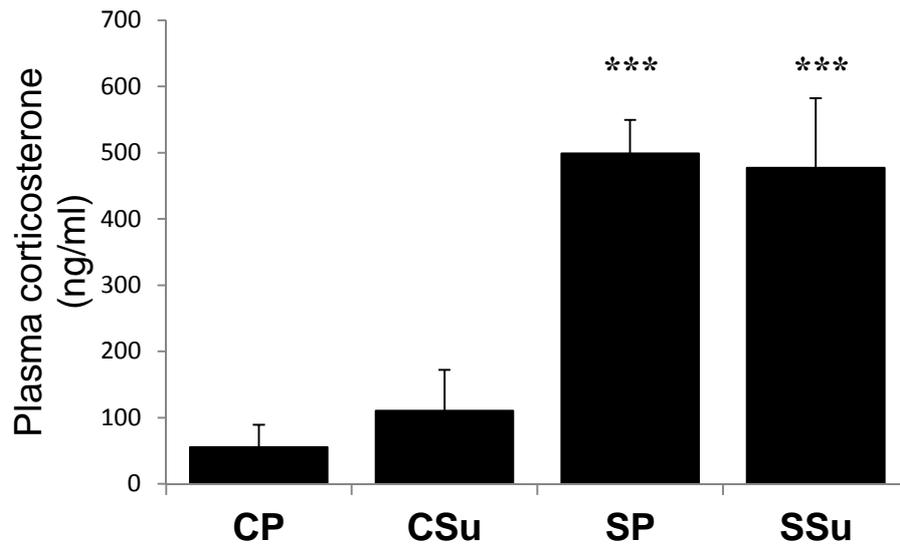
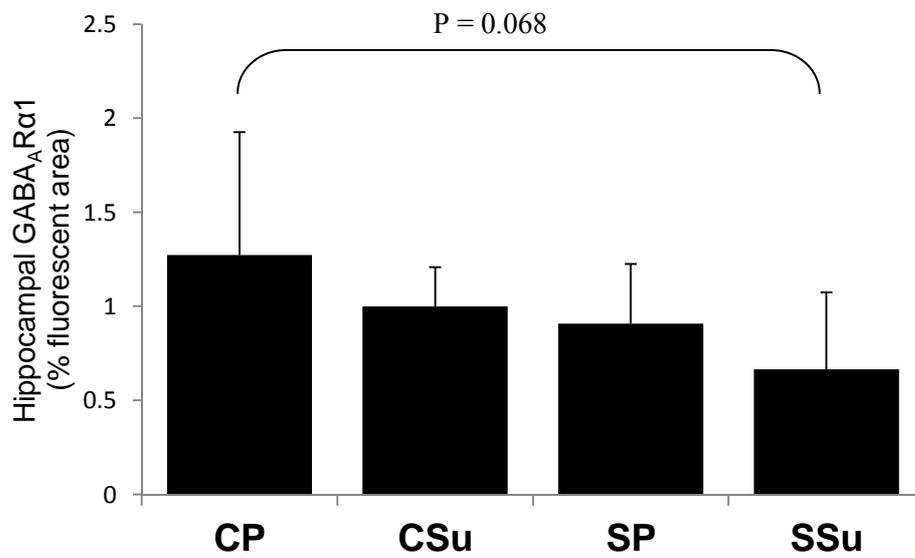


Figure 4.2. Effect of acute restraint stress and/or of *S. frutescens* on plasma corticosterone concentrations, n = 10. Abbreviations: CP, control placebo, SP, stress placebo, CSu, control *S. frutescens*-treated, and SSu, stress *S. frutescens*-treated.
 ***: P < 0.0001, different from control groups

Immunohistochemistry data illustrated a tendency of hippocampal GABA_ARα1 to decrease in the presence of both stress and *S. frutescens* administration (P = 0.068; Fig. 4.3a). Hippocampal GABA_ARα1 levels in CA1 regions decreased by 21.5% after *S. frutescens* administration, 28.8% after exposure to restraint and 47.8% after both *S. frutescens* administration and restraint. Although these changes were not statistically significant, they may have clinical importance. Representative photos taken of the CA1 regions of the hippocampus are shown in Fig 4.3b.

Hippocampal GR showed no significant effects of either stress or *S. frutescens* administration, likely due to high variability seen in control groups (Fig 4.4a). However, restraint caused an average 39.8% decrease in hippocampal GR in placebo-treated groups and a 47.9% decrease after *S. frutescens*-treatment. Representative photos of the CA1 regions of the hippocampus and are shown in Fig 4.4b.

(a)



(b)

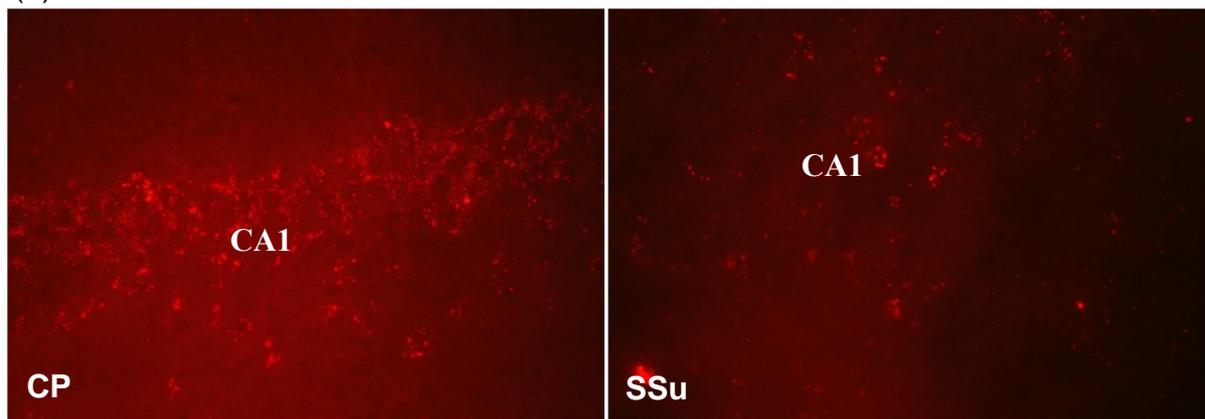
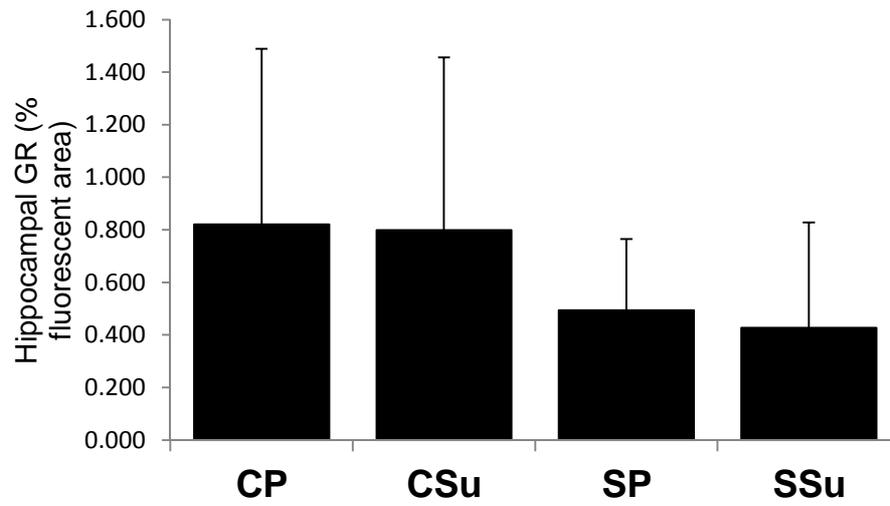


Figure 4.3. Effect of acute restraint stress and/or *S. frutescens* on hippocampal GABA_AR α 1, n = 4. Abbreviations: CP, control placebo, SP, stress placebo, CSu, control *S. frutescens*-treated, and SSu, stress *S. frutescens*-treated shown in (a), and representative samples of CP and stress SSu rat hippocampal sections (magnification 400x) visualized for GABA_AR α 1 shown in (b).

(a)



(b)

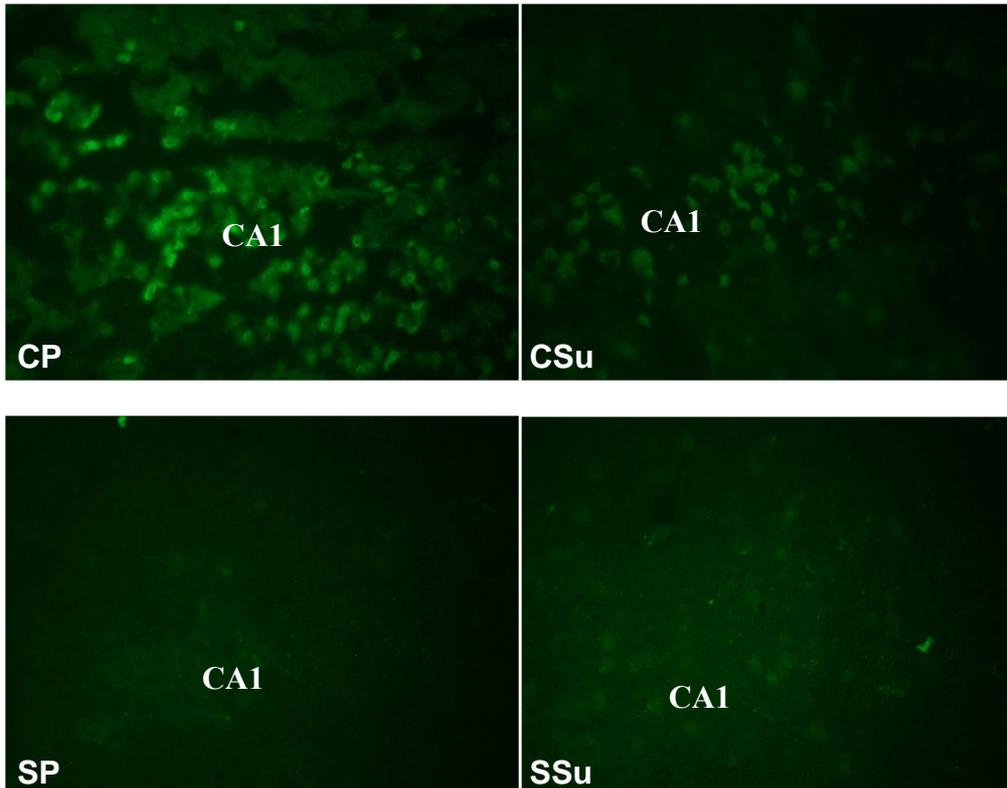


Figure 4.4. Effect of acute restraint stress and/or *S. frutescens* on hippocampal GR, $n = 4$. Abbreviations: CP, control placebo, SP, stress placebo, CSu, control *S. frutescens*-treated, and SSu, stress *S. frutescens*-treated shown in (a), and representative samples of CP, CSu, SP, and SSu rat hippocampal sections (magnification 400x) visualized for GR shown in (b).

ACTH levels in the anterior pituitary show a gradual increase brought on by *S. frutescens*-treatment and restraint stress independently as well as cumulatively (Fig 4.6). *S. frutescens*-treatment led to a 51.1% increase, stress led to a 206.1% increase and a combination of the two led to a 357.6% increase in ACTH levels, with SP > CSu (P < 0.05).

No statistically significant effect of stress on circulating ACTH concentrations were seen in placebo groups (Fig 4.7), despite a major increase in SP vs. CP – this was probably due to increased variation in SP. Statistically significant differences in ACTH levels were also found between stress and control groups treated with *S. frutescens* where stressed rats showed higher concentrations than control rats treated with either placebo or *S. frutescens* (P < 0.05). These stress-induced effects, which occur only in the presence of *S. frutescens*, point to a possible interaction effect between stress and *S. frutescens*. A noteworthy tendency towards increased ACTH levels in SSu vs. SP (P = 0.074) was also observed. A relatively high inter-individual variation was observed in both SP and SSu for this parameter. Lower detection limit for this assay was 1.5 pg/ml.

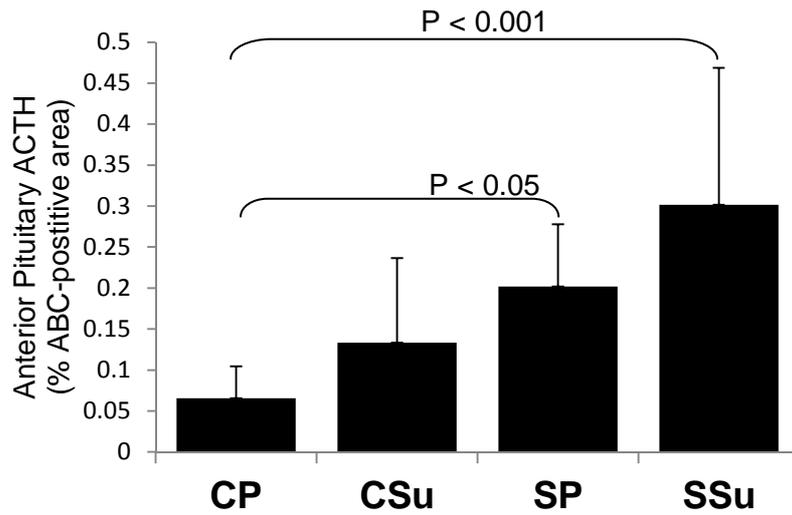


Figure 4.5. Effect of acute restraint stress and/or of *S. frutescens* on anterior pituitary ACTH concentrations, n = 4. Abbreviations: CP, control placebo, SP, stress placebo, CSu, control *S. frutescens*-treated, and SSu, stress *S. frutescens*-treated.

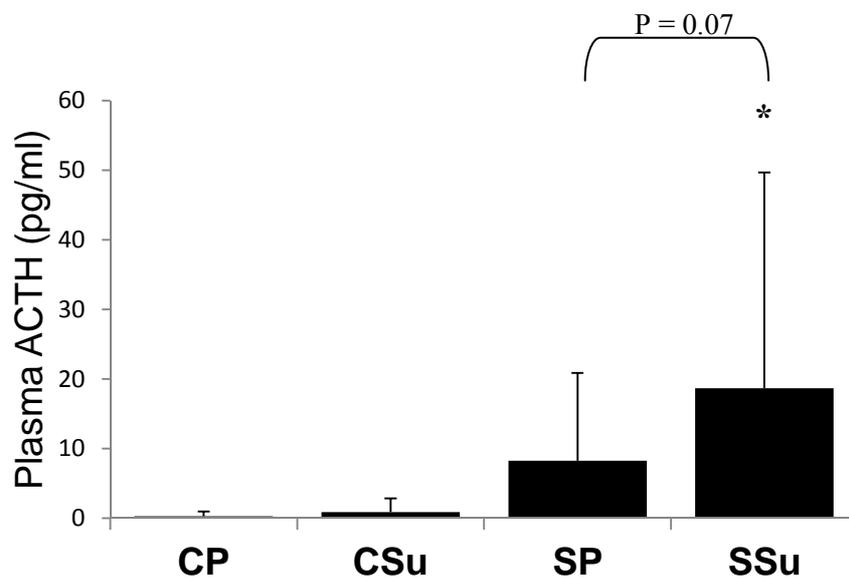


Figure 4.6. Effect of acute restraint stress and/or *S. frutescens* administration on plasma ACTH concentrations, n = 10. Abbreviations: CP, control placebo, SP, stress placebo, CSu, control *S. frutescens*-treated, and SSu, stress *S. frutescens*-treated.

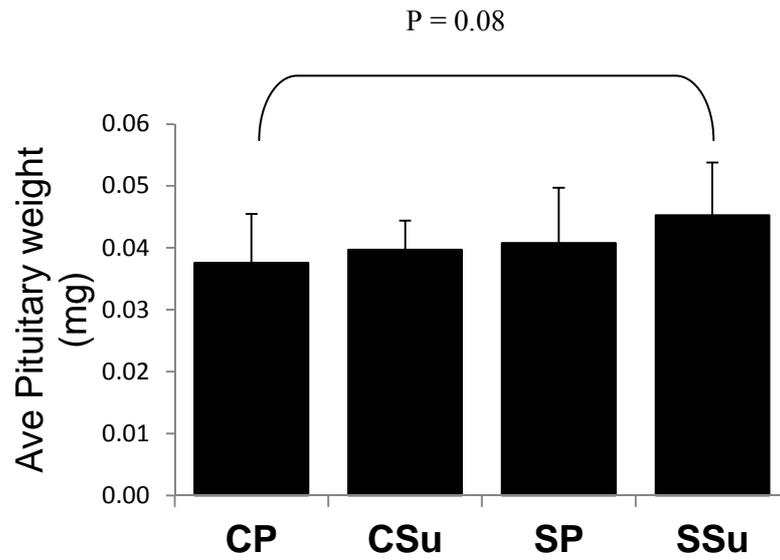
*: P < 0.05, different from CSu and CP.

No statistically significant effects of either stress or *S. frutescens* alone were seen in pituitary mass. However, with exposure to both stress and *S. frutescens*, rat pituitary mass tended to be higher. (SSu > CP; P = 0.08; Fig 4.8a). Adrenal mass showed a significant decrease in CSu that was attenuated in response to stress (P < 0.05; Fig 4.8b).

Adrenal levels of ACTH showed a reciprocal trend to pituitary and circulating ACTH levels with SSu showing sharp decreases (P < 0.05) and SP showing a tendency (P = 0.075) compared to CP animals (Fig 4.9a). No statistically significant differences were seen in adrenal IL-6, increases were seen with administration of *S. frutescens* (38.8%), stress exposure (43.3%) and a 58.2% increase seen with both *S. frutescens*-treatment and stress exposure (Fig 4.9b).

Neither stress, nor *S. frutescens* administration had any effect on serum testosterone levels. Average testosterone concentrations were 9.27 ± 0.42 ng/mL for CP, 10.38 ± 2.02 ng/mL for SP, 10.56 ± 2.21 ng/mL for CSu and 9.52 ± 1.56 ng/mL for SSu. Lower detection level for this assay was 83 pg/ml.

(a)



(b)

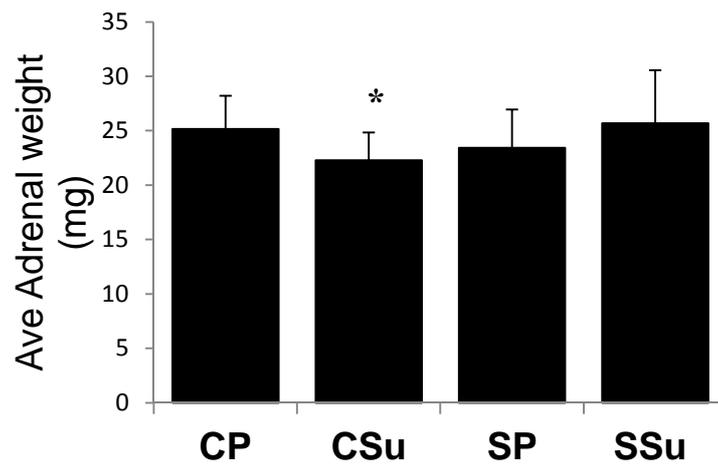
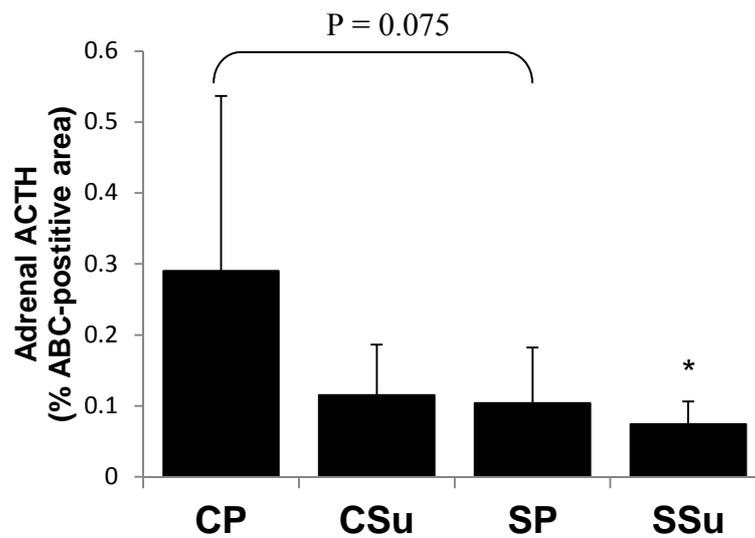


Figure 4.7. Effect of acute restraint stress and/or *S. frutescens* on (a) pituitary mass and (b) average adrenal mass, n = 10. Abbreviations: CP, control placebo, SP, stress placebo, CSu, control *S. frutescens*-treated, and SSu, stress *S. frutescens*-treated.

*: P < 0.05, different from CP and SSu.

(a)



(b)

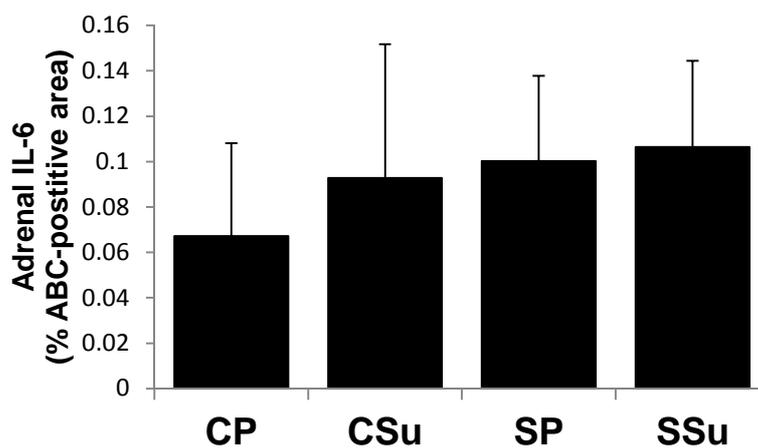


Figure 4.8. Effect of acute restraint stress and/or *S. frutescens* on (a) adrenal ACTH and (b) adrenal IL-6, n = 4. Abbreviations: CP, control placebo, SP, stress placebo, CSu, control *S. frutescens*-treated, and SSu, stress *S. frutescens*-treated.

*: P < 0.05, different from CP

4.4 Discussion

The current study yielded the following main findings: A major increase in plasma corticosterone levels was seen after exposure to restraint stress that was not significantly affected by acute *S. frutescens*-administration. A tendency of GR levels to decrease in response to restraint as well as a subtle decrease in GABA_AR levels was also seen in the CA1

region of the hippocampus. This is thought to be achieved synergistically by exposure to restraint stress and *S. frutescens* treatment. Thus, contrary to our hypothesis, *S. frutescens* did not lead to an attenuated psychological stress response by increased GABAergic function. A subtle tendency of pituitary mass to increase with the combination of stress and *S. frutescens*-treatment was observed which coincided with an increase in anterior pituitary ACTH levels in SSu animals. Plasma ACTH levels increased in response to stress and this increase also seemed to be exacerbated in the presence of *S. frutescens*. Decreased adrenal mass was seen in response to treatment with *S. frutescens*, this decrease was attenuated by stress exposure. A significant decrease in adrenal ACTH levels in response to both stress and *S. frutescens*-treatment was seen. The consistent patterns observed throughout the results, albeit subtle for some parameters, affirm a tendency for causality between the separate changes.

As reported in Chapter 3, acute restraint once again resulted in a significant increase in plasma corticosterone levels. However, contrary to previous studies performed by our group using a chronic supplementation model of intermittent restraint stress (Prevoe *et al.*, 2004), *S. frutescens* had no effect on corticosterone production during acute stress. Since the acute model was chosen in order to elucidate the central effects of *S. frutescens*-administration it may not be ideal for analyzing the peripheral effects. Corticosterone release occurs within minutes of stress perception (Hess *et al.*, 1968) and could precede the effects of *S. frutescens* since enough time may not have passed for the *S. frutescens* to become bioavailable. Corticosterone also has a half-life of 20 to 25 minutes in male rats (Kitay, 1961, Sainio *et al.*, 1988) and would still be detectable at the time point of sacrifice which is likely why no effect of *S. frutescens* on circulating corticosterone concentrations was seen in the acute model.

The lack of statistically significant differences between groups with regard to GR could be attributed to large variability seen in control groups, indicating variable baseline concentrations in control groups. This variability is likely due to the social hierarchy that exists between rats housed together in a cage, resulting in lower basal GR levels in socially submissive animals relative to dominant animals. Chronic social stress has been shown to induce steroid induce nerve growth factor (NGF) release, which functions as a GR inhibitor, and eventually cause steroid insensitivity (Sheridan *et al.*, 2000). This result, however, is unavoidable in a stress model, since housing rats separately would lead to isolation stress (Hatch *et al.*, 1963) which would also influence the results. The variability seen in control groups seem to decrease in SP rats indicating a more uniform response of GR to decrease with psychological stress-induced corticosterone increases.

Another possibility that could explain the trend seen for GR to decrease in response to stress, is the conformational changes which occur once the GR binds a ligand. The GR antibody was raised against 300 residues lying in an internal region of the protein which may undergo conformational changes and therefore mask the epitope resulting in lower signals being obtained for the complexes. In addition, compounds found within *S. frutescens* which are structurally similar to corticosterone may also complex with the GR, resulting in a lowered signal. This could also result in downstream effects similar to the natural ligand, possibly explaining the raised corticosterone levels seen in CSu.

The tendency of hippocampal GR to decrease in response to stress is supported by previous studies by our group and others which showed similar result in a chronic model of restraint in liver tissue (Omrani *et al.*, 1980, Sapolsky *et al.*, 1984, Smith *et al.*, 2007). It has been shown that chronic stress causes atrophy in the CA3 region of the hippocampus (McKittrick *et al.*, 2000), while both chronic as well as acute stress leads to suppression of neurogenesis in rats. This psychological stress-induced damage results in cognitive impairment in the learning of

spatial and short-term memory tasks (McEwen and Sapolsky, 1995). These detrimental effects are known to be caused by stress-induced corticosterone secretion and binding to GR (Pavlidis *et al.*, 1994, McEwen and Sapolsky, 1995, Pavlidis *et al.*, 1995, Cameron and Gould, 1996, Conrad *et al.*, 1996, Pavlidis *et al.*, 1996, Gould *et al.*, 1997, de Kloet *et al.*, 1998b). Our result is novel in that it is the first time that this well characterized adaptation of GR to *chronic* psychological stress (Magarinos *et al.*, 1996) has been illustrated after acute exposure to stress.

It has been shown that orally ingested GABA remains intact through the absorption process and is capable of crossing the blood brain barrier (Kuriyama and Sze, 1971). The anecdotal mood altering effects of *S. frutescens* along with its GABA content (Tai *et al.*, 2004) would suggest an ability to increase GABA content, resulting in increased inhibitory neurotransmission function of GABAergic neurons. Since acute stress has been shown to down-regulate GABA receptor function (Barbaccia *et al.*, 1996), it would be expected that *S. frutescens* administration would attenuate the stress-induced GABA down-regulation. Attenuation of stress-induced GABA down-regulation is expected to result in a continued GABA_AR activity and GABAergic function within the CA1 region of the hippocampus. However, the tendency for hippocampal GABA_AR to decrease in the presence of both psychological stress and *S. frutescens* suggests that the combination of acute stress exposure and *S. frutescens* is adequate to induce this slight down-regulation of GABA_AR. These conclusions are however based on tendencies and a larger sample size will be required to confirm our suspicions.

A possible explanation for the tendency of GABA_AR levels in the hippocampus to decrease could be the bipolar nature of the GABA neurotransmitter. Recent evidence demonstrated that GABA may transiently act as an excitatory transmitter after intense bursts of GABA_AR activation in the brain. This is due to an activity-dependent shift in chloride reversal potential

which causes a reversed polarity of GABAergic postsynaptic responses (Isomura *et al.*, 2003). This phenomenon has been observed in the mature CA1 region of the hippocampus (Fujiwara-Tsukamoto *et al.*, 2003) and likely occurs because of the fine and complex structure of neurons. Asymmetric intracellular Cl⁻ activities caused by variations in Cl⁻ fluxes through ion channels or by asymmetric expression and activities of Cl⁻ transporters are thought to cause the change in function of GABAergic synapses from hyperpolarization to depolarization (Walters, 2004). The prolonged activation of GABA brought on by the addition of external plant-derived GABA, could therefore bring about this functional shift, depolarizing presynaptic glutamatergic nerve terminals projecting to hypothalamic neurons, leading to subsequent excitatory glutamate release (Jang *et al.*, 2001). The addition of *S. frutescens*-derived GABA in our model may thus have resulted in a burst in GABA activity in the hippocampus shortly after administration, leading to a phenomenon known as “afterdischarge” (persistence of response of neural elements after cessation of stimulation) (Millhorn *et al.*, 1980), where a functional shift in GABAergic neurons causes the depolarization of glutamatergic neurons and excitatory neurotransmission. The saturation of GABAergic receptors could therefore lead to a down-regulation of GABA_AR. This is supported by the tendency for decreased GABA_AR seen in the hippocampal CA1 region.

Inhibition of GABA_AR mediated synaptic transmission has also been observed in hippocampal CA1 neurons after acute cannabinoid administration (Katona *et al.*, 1999). Cannabinoids such as tetrahydrocannabinol (THC) are the primary mood-elevating elements found within marijuana, a shrub which is also known for its anxiolytic effects. The inhibitory effects of cannabinoids on GABAergic neurons are shown to be mediated through cannabinoid-receptor 1 (CB1) and likely involves down-regulation of presynaptic voltage-dependent Ca²⁺ channels (Hoffman and Lupica, 2000, Katona *et al.*, 2001, Wilson *et al.*, 2001, Wilson and Nicoll, 2001). Even though the exact mechanisms of this CB1 mediated

inhibition of hippocampal GABAergic neurons are still unknown, it could be postulated that similar mechanisms could be at work after *S. frutescens* administration since it shows a tendency for GABA_AR to decrease in hippocampal CA1 regions- an effect shown to be present after acute marijuana administration as well. It is possible that *S. frutescens* activates an endogenous cannabinoid (endocannabinoid) pathway that mimics the effects of marijuana use. It is also possible that *S. frutescens* mediates its anxiolytic effects in a similar fashion as marijuana, which is postulated to decrease inhibitory GABAergic neurotransmission in the hippocampus and amygdala, causing an increased glutamatergic outflow which in turn activates the mesolimbic dopamine pathway (the primary reward pathway) (Nestler and Carlezon, 2006). These effects may be perceived differently between species, and while humans perceive the effect as anxiolytic, rats may perceive the experience as intoxication and therefore might experience a psychological stress response. Further studies examining the effects of *S. frutescens* administration prior to an acute stress response in humans, using a model of public speaking for example, is needed to shed more light on this matter.

This theory is supported by the tendency for mass-increases seen in pituitary glands of rats exposed to both stress and *S. frutescens* treatment ($P = 0.078$). Neither *S. frutescens*, nor stress alone had a significant effect on pituitary mass. Exposure to acute restraint stress combined with the *S. frutescens*-induced increase in neuronal excitation of the hypothalamus, likely lead to an increase in pituitary activation and the subsequent tendency of pituitary hypertrophy seen in SSu.

The tendency for decreased GABAergic activity within the hippocampus as well as decreased pituitary mass is mirrored by pituitary ACTH production as seen in results of anterior pituitary analysis –known to be the primary site of ACTH production. This concurs with literature, where the effects of hippocampal lesions as well as hippocampalectomies on ACTH levels were used to establish the role of the hippocampus in the regulation of the HPA

axis (Hershenson and Moos, 1986). The reciprocal relationships seen between GABAergic function and ACTH production therefore suggests that decreased GABAergic output from the hippocampus, induced synergistically by restraint stress as well as *S. frutescens* administration, lead to an inverse ACTH production and release.

Furthermore, stressed animals treated with *S. frutescens* tended to have an increased plasma ACTH concentration when compared to stressed animals treated with placebo (SP) ($P = 0.074$). These results coincide with the above-mentioned *S. frutescens*-mediated activation of the pituitary gland, and are likely the result of the same central neuronal activation. The lack of significant increases in plasma ACTH levels seen in SP animals could be attributed to an intact corticosterone-mediated negative feedback loop as well as the relatively short half-life of ACTH in circulation.

Adrenocortical ACTH levels seem to show an inverse gradient to circulating ACTH concentrations. The lack of differences seen between group levels of adrenocortical IL-6 could likely be attributed to the structural properties of the antibody used in IL-6 detection. Studies utilizing size exclusion chromatography and equilibrium centrifugation have shown that IL-6 binds to the soluble extracellular C-terminal domain of IL-6R (Ward *et al.*, 1994). The antibody used to detect IL-6 was directed to bind the C-terminal as well, resulting in only free IL-6 being detected by the ABC staining. Since IL-6 acts in an auto-/paracrine manner to stimulate glucocorticoid production (Arzt *et al.*, 1998), it has its effects in close proximity from the point of release and free IL-6 would not give an accurate representation of the functional state of IL-6 in the adrenal cortex.

The phenomenon of sympathetic GABAergic input has been observed in the splanchnic nerves originating from the spinal cord, innervating adrenal medulla and cortical cells since the late eighties (Peters *et al.*, 1989). It is therefore plausible to argue that acute

administration of GABA-rich *S. frutescens* leads to an over-activation of central and peripheral GABA_AR, causing the functional shift mentioned earlier. Even though the exact mechanisms are still unknown, the inhibition of adrenal corticosterone production in response to stress observed during chronic administration of *S. frutescens* could likely be attributed to a chronic GABAergic activation in the neuronal milieu. Autofluorescence, possibly due to faulty fixation, compromised the analysis of GABA_AR in the adrenal gland. Investigation of GABA_AR in the adrenal gland could elucidate the degree of neuronal excitation and *ergo* sympathetic contribution to adrenocortical function. This constitutes a potential avenue of investigation for future studies.

Even though the raised plasma corticosterone levels did not differ between stressed rats treated with *S. frutescens* or placebo, plasma ACTH showed significant increases in stressed rats receiving *S. frutescens*. (This evidence suggests that the HPA-axis was only sufficiently activated during a state of exacerbated sympathetic output from the hippocampus to the hypothalamus). It seems that at a time point of one hour after the onset of an acute mild psychological stressor, the major stimulatory input to adrenocortical cells to produce and secrete corticosterone arises from sympathetic splanchnic nerves, as suggested in Chapter 3.

These findings are in agreement with a vast body of literature. The discrepancies between circulating plasma levels of ACTH and corticosterone have been apparent since the late fifties (Liddle *et al.*, 1959). It is only recently, however, that the sympatho-adrenal medullary system has been brought to the forefront of stress physiology. Traditionally the HPA axis was thought to play the primary role in adrenocortical regulation (Plotsky *et al.*, 1998). This view has been challenged using modern retrograde fiber tracing techniques providing reports of splanchnic nerves terminating in the adrenal cortex (Kesse *et al.*, 1988, Afework, 1989, Coupland *et al.*, 1989). Even though traditionally only the adrenal medulla was associated with innervations (Bornstein and Chrousos, 1999), evidence now suggests the presence of a

smaller quantity of branched neural fibers also distributed into the cortex (Parker et al., 1993).

Conducting the experiment during a reversed light/dark cycle led to an increase in basal and stressed corticosterone levels in the current study. These increases are likely due to the rat's circadian rhythm which increases corticosterone secretion during active times of the day.

The importance of sympathetic regulation of the adrenal cortex, in the context of the acute stress response, is once again demonstrated by the lack of left adrenal hypertrophy found during a reverse light/dark cycle in the current study (restraint during dark). The rationale behind the reversing of the light-dark cycle was to provide an awake state in rats (which are known to be nocturnal) during the conduction of the experimental protocol, thereby maximizing perception and awareness of the specific intervention. Conducting the experiment during a reversed light/dark cycle led to an increase in basal and stressed corticosterone levels in the current study relative to the previous study performed (56 ± 33 ng/ml vs. 7 ± 3 ng/ml). These increases are likely due to differences between batches of rats or the rat's circadian rhythm which increases corticosterone secretion during active times of the day. The circadian regulation of adrenal glucocorticoid secretion has been well established in vertebrates (Bartter *et al.*, 1956). This diurnal cycle is characterized by persistence in the absence of stimuli and remains intact in blind patients (Landau and Feldman, 1954). The sympathetic input to the adrenal gland has also been shown to be sensitive to photo-stimulation. In rats, periods of darkness result in a dramatic increase in norepinephrine secretion from sympathetic nerves (Hashimoto, 1999). Since norepinephrine is a powerful activator of the adrenal medulla chromaffin cells and subsequent cortical steroidogenesis, the lack of left adrenal hypertrophy seen in the results of the current study could be attributed to higher "background noise" produced by a increased basal drive of the sympathetic nervous system when compared to Chapter 3. From this data, we can make the

following recommendation: a normal light/dark cycle (i.e. interventions during the light phase) is recommended for studies concerning the sympatho-adrenal medullary system.

A statistically significant decrease in average adrenal mass was seen in *S. frutescens*-treated rats not exposed to stress. This effect of *S. frutescens* was attenuated in response to stress. In contrast to its effect on adrenal mass, *S. frutescens* lead to a tendency for circulating corticosterone concentrations to increase (96.4% in controls). These seemingly opposing effects can be explained: the decrease in adrenal mass in controls may simply be the result of liposome depletion as a result of mildly increased corticosterone production known to occur in response to *S. frutescens*. A decreased liposome content is indeed recognised as a histological indicator of the acute stress response (von Euler, 1967), as also discussed in Chapter 3. Furthermore, the metabolic effect of *S. frutescens* to increase the glucocorticoid levels is facilitated by its effect to decrease plasma glucose levels (Chadwick et al., 2007). This decrease in plasma glucose levels, in the presence of a relatively low level corticosterone environment, could lead to an increased demand for free fatty acids across the body, decreasing the bioavailability of free fatty acids to replenish liposome stores in the adrenal cortex, further contributing to the decreased adrenal mass reported. The fact that adrenal mass did not decrease further in stressed animals, can be explained by the counteracting effects of substantial increases in corticosterone levels. Firstly, adrenal cortices would undergo large scale hypertrophy and/or hyperplasia in response to stress (as demonstrated in Chapter 3), adding to organ mass. Secondly, high levels of circulating corticosterone would have resulted in significantly increased gluconeogenesis, enabling more free fatty acids to replenish adrenal liposome stores.

No significant variation in plasma testosterone levels were seen between groups, either after stress exposure, or *S. frutescens* administration. Literature reports acute restraint stress leading to decreased plasma LH and testosterone assessed at various time points after stress

exposure, however these models likely induced a more severe psychological stress response (Sapolsky and Krey, 1988, Johnson *et al.*, 1992, Norman and Smith, 1992). The lack of significant variation seen in testosterone levels in response to *S. frutescens* administration suggests that no androgen-related side effects would be encountered during the clinical application of *S. frutescens*.

This exacerbated stress response, likely due to the tendency of GABA_AR to decrease in SSu, caused the activation of both the HPA-axis and the sympatho-adrenal medullary system (Fig. 4.10). It is noteworthy that even though no differences were seen in plasma corticosterone levels between stress groups receiving placebo and those receiving *S. frutescens*, plasma as well as pituitary ACTH levels only increased significantly rats treated with *S. frutescens*. We can therefore assume that the ACTH-mediated HPA-axis was only sufficiently activated during the exacerbated stress response and that glucocorticoid regulation in a typical psychological stress response would be primarily under the control of the sympatho-adrenal medullary system.

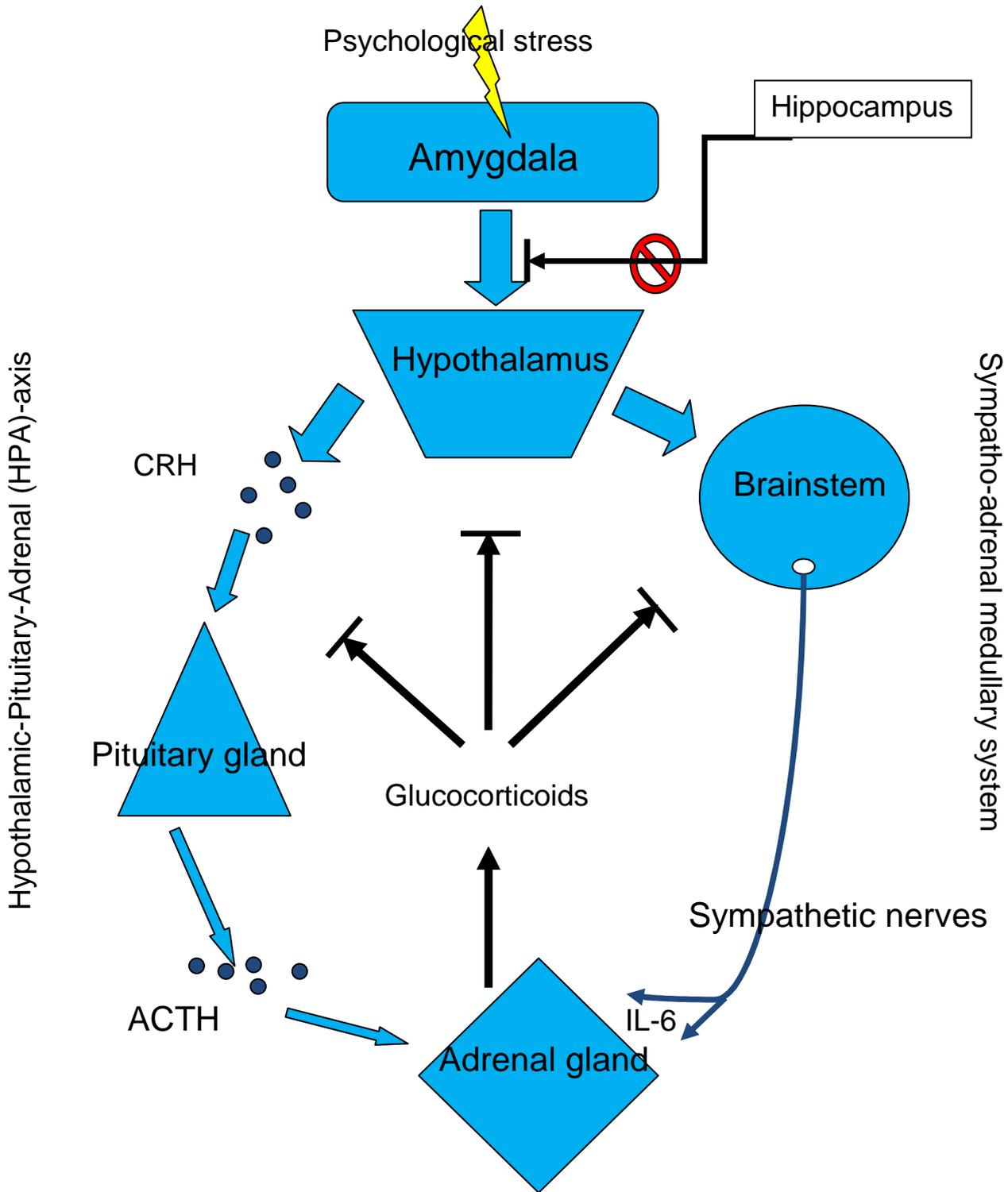


Figure 4.9. Schematic representation of the pathways involved in stress signal transduction 90 minutes after *S. frutescens* administration with blue indicating activated pathways.

Chapter 5

Conclusion

From data presented in Chapter 3, we can conclude that IL-6 plays an important role in the sympatho-adrenal medullary system, and that blocking this pathway effectively attenuates the corticosterone response to acute psychological stress. Taken together, these results suggest that in the immediate response to acute stress, the stimulatory effect on the adrenal cortex to initiate glucocorticoid release is under direct neural control, facilitated through the sympatho-adrenal medullary system. Furthermore, the data suggest that the systemic endocrine signal produced by the HPA-axis is an alternative pathway for glucocorticoid release initiated somewhat later in the response to stress. We can therefore accept our first hypothesis.

Furthermore, the phenomenon of afterdischarge, brought on by the combination of stress and *S. frutescens* administration, has significant implications for stress-relief treatment strategies involving acute administration of GABA. Data presented in Chapter 4 demonstrated that acute *S. frutescens*-administration lead to a tendency for decreased inhibitory output of the hippocampus, creating an augmented stress response that was sufficient to activate both the sympatho-adrenal medullary system as well as the secondary HPA-axis. Given this result, we conclude that the anxiolytic effects of *S. frutescens* anecdotally reported in humans is most likely dependent on activation of the mesolimbic dopaminergic reward pathway. Furthermore, given previous reports of an effect of *S. frutescens* to partially inhibit steroidogenesis at adrenal level, we conclude that in our model, time point of sacrifice was probably too early to illustrate these proven effects at peripheral target tissue level. We therefore reject our second hypothesis, since acute *S. frutescens*-administration did not show any central anxiolytic effects.

In terms of the potential use of *S. frutescens* as clinical anxiolytic treatment, we have some reservations. Although there is convincing evidence in the literature suggesting a beneficial effect of chronic treatment, acute treatment actually aggravated the stress response in our model. Although we acknowledge that this increased stress response may have been an “artifact” of having an animal model, one cannot disregard the possibility of a similar effect in humans. Therefore, our recommendation is that clinical studies be performed in humans, so that the central responses can also be assessed subjectively, e.g. by questionnaire. This should be done in combination of personality classifications, to more clearly define which populations may in fact benefit from *S. frutescens* treatment, and which may show a negative reaction to treatment, which could manifest as feelings of paranoia and anxiety, and in fact exacerbate the pre-existing pathology or stress status.

Lastly, future studies investigating shifts in GABAergic activity within the adrenal glands and amygdala after exposure to restraint stress and/or acute *S. frutescens* administration could further elucidate the excitatory vs. inhibitory effects of *S. frutescens* treatment.

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Appendix A

Cytokine Bio-plex multiplex bead-based assay

To determine the plasma cytokine concentrations, a commercially available bead-based system, obtained from Biorad Laboratories Inc. (Parklands, South Africa), was used. This kit system comprised the following components:

- 171-K11070 - Multiplex kit, including cytokine antibody-coupled beads (specific for cytokines specified by user), detection antibodies and standards
- 171-305008 - Serum diluent specific for rat samples
- 171-203001 - Reagent kit, including 96-well filter plate, assay buffer, wash buffer, detection antibody diluent, PE-labelled streptavidin and sealing tape.

NOTE: In order to maintain the Bioplex instrument, regular calibration and validation of day-to-day reproducibility was performed by a laboratory technician (using calibration kit with catalogue number 171-000201 and validation kit with catalogue # 171-203060).

Procedure:

1. Pre-wet filter plate with 100 μ l of Bio-Plex assay buffer.
2. Vortex multiplex bead solution and add 50 μ l into each well.
3. Wash twice with 100 μ l wash buffer (remove buffer by vacuum filtration).
4. Add 50 μ l standards, controls and samples (prepared according to manufacturers' instructions) into designated wells, cover with sealing tape and aluminium foil and incubate on microplate shaker at 300 rpm for 30 min at RT.
5. Wash three times with wash buffer.
6. Add 25 μ l detection antibody (prepared according to manufacturers' instructions), cover and incubate on microplate shaker at 300 rpm for 30 min at RT.

7. Wash three times with wash buffer.
8. Add 50 μ l streptavidin-PE, cover and incubate on microplate shaker at 300 rpm for 10 min at RT.
9. Wash three times with wash buffer.
10. Resuspend the beads in each well with 125 μ l of Bio-Plex assay buffer and read immediately on Bio-Plex system.

Appendix B

Enzyme immuno assay (EIA)

Plasma corticosterone (AC-14F1, Immunodiagnostic systems), ACTH (7023, Biomerica) and testosterone (EIA-1559 DRG Diagnostics) was assayed according to the manufacturer's instructions. Assays were carried out using 96-well micro titre plates coated with either a polyclonal rabbit anti-corticosterone antibody or a mouse monoclonal anti-testosterone antibody. All reagents, including antibody reagents, used was supplied with the kit.

Procedure:

1. Add 100 μ l standards, controls (non-serum, provided in kit) and samples in duplicate in the appropriate wells.
2. Add 100 μ l enzyme conjugate solution in each well and mix. For the corticosterone assay. The solution contained either HRP labelled corticosterone or testosterone. Incubate for 1 hr at RT.
3. Shake out the contents and wash three times with 400 μ l PBS-Tween. Blot dry on absorbant tissue.
4. Add 200 μ l substrate solution TMB)/H₂O₂ and incubate 30 min at RT.
5. Add 100 μ l stop solution (0,5M HCl or 0.5M H₂SO₄) to all wells.
6. Measure absorbance at 450 nm within 30 min.

Prepare the standard curve and determine the sample concentrations

Appendix C

Immunohistochemistry (IHC) using fluorescence

Co-staining were performed using primary antibodies directed against GABA_AR α 1 and GR with Texas Red and FITC secondaries used respectively for visualisation. Specific antibodies used in the current experiment included:

- a) GABA_AR α 1 assay. Prim Ab, 1:1000 (sc-31404, Santa Cruz Biotechnology Inc.); sec Ab, 1:1000 (sc-2090, Santa Cruz Biotechnology Inc.).
- b) GR assay. Prim Ab, 1:1000 (sc-8992, Santa Cruz Biotechnology Inc.); sec Ab, 1:1000 (sc-2783, Santa Cruz Biotechnology Inc.).

Procedure:

1. Fix frozen sections in 2% paraformaldehyde for 10 min at RT.
2. Wash slides in 0.1 M PBS.
3. Encircle samples with a wax pen.
4. Block for 30 min in 5% serum at RT. (*Note: use the same serum in which the secondary antibody is raised*)
5. Shake off serum and incubate sections for 4 hr at RT or overnight at 4°C with the 1st primary antibody. (*Note: Do not wash after serum blocking step*)
6. Wash slides with PBS and add the secondary antibody to the sections. Incubate for 1 hr at RT. (donkey anti-goat in the case of GABA_AR α 1 and donkey anti-rabbit in the case of GR). (*Note: the secondary antibody is fluorescently labelled, so from this step forward, all steps should be performed in the dark*)

7. Wash slides with PBS and add the 2nd primary antibody overnight at 4°C or for 4 hrs at RT, depending on the 1st primary.
8. Add the secondary antibody for 1 hr after washing the sections thoroughly with PBS.
9. Wash sections and add Hoechst (1/8000) for 15 min.
10. Wash slides well and mount with DAKO fluorescent mounting medium (Dako, Diagnostech, Glostrup, Denmark).

Appendix D

Immunohistochemistry (IHC) using ABC method

- All steps are carried out at RT in a humidified chamber.
- Slides are placed horizontally for staining. Ensure application of sufficient volumes of reagents to completely cover the sections; 100 µl is usually adequate, or 1-3 drops of working solutions. Use filter paper to remove reagents after each step but avoid drying of samples between steps.

Antibodies used included:

- a) IL-6 assay. Prim Ab, 1:800 (sc-1265, Santa Cruz Biotechnology Inc.); sec Ab, 1:800 (sc-2023, Santa Cruz Biotechnology Inc.).
- b) ACTH assay. Prim Ab, 1:800 (NB 600-1367, Novus Biologicals); sec Ab, 1:800 (sc-2017, Santa Cruz Biotechnology Inc.)

Deparaffinise and dehydrate paraffin embedded tissue prior to immunohistochemistry

Incubate sections in xylene for 2 changes of 5 min each, 100 % absolute alcohol for 2 changes of 3 min each, 95 % alcohol for 2 changes of 3 min each, 80 % alcohol for 3 min, 50 % alcohol for 3 min, and then in distilled water for 2 changes, 3 min each.

Procedure:

1. Incubate slides for 5-10 min in 1% H₂O₂ diluted in 0.1 M PBS to quench endogenous peroxidase activity and wash slides twice with PBS.
2. Reduce non-specific (background) staining by blocking for 1 hr in 1.5% serum in PBS.

3. Incubate with primary antibody for 30 min at RT or overnight at 4° C. (Optimal concentration should be determined for each experiment by titration; recommended range is 0.5-5.0 µg/ml, diluted in 1.5% blocking serum).
4. Wash three times with 0.1 M PBS.
5. Incubate sections for 30 min with biotinylated secondary antibody at approximately 1 µg/ml and wash three times with PBS.
6. Incubate sections for 30 min with AB enzyme reagent and wash three times with 0.1 M PBS.
7. Incubate sections in 1-3 drops peroxidase substrate for 3 sec – 10 min or until desired stain intensity develops. The section may be checked for staining by rinsing with H₂O and viewing under a microscope. If necessary, add additional peroxidase substrate and continue to incubate. Standardise this incubation time for each experiment once optimized.
8. Wash five times with deionised H₂O.
9. Counterstain with Gill's formulation #2 hematoxylin for 5-10 sec and wash with deionised H₂O.
10. Destain with 1% acid alcohol (10 ml 1 % HCl dissolved in 1 l 70 % alcohol) and bluing reagent (supplied in kit) and wash with tap water.
11. Dehydrate by dipping slides 2X in 95% ethanol for 10 sec each, 2X in 100% ethanol for 10 sec each and 3X in xylene for 10 sec each
12. Immediately add 1-2 drops of permanent mounting medium and cover with glass coverslip.