THE EFFECTS OF PHYSICAL AND PSYCHOLOGICAL STRESS ON THE BEHAVIOUR AND NEUROCHEMISTRY OF RATS

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DECLARATION:

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

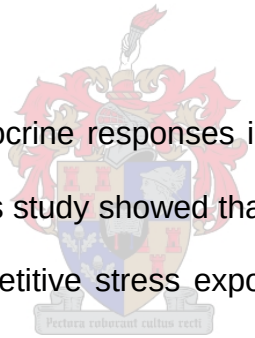
Signature: ……..Date:…….

SUMMARY

Stress is considered one of the major factors involved in the pathogenesis of affective disorders, for example, direct and indirect exposure to terrorist attacks or being subjected to subtle victimization. There is a long history of development of procedures to determine anxiety responses in animals in order to find new or better treatments for patients. Prior stress exposure is known to alter the activation response to a subsequent stressor and the means of coping with stress can influence health and disease. This orchestrated process, usually referred to as the "stress response", involves various mechanisms, which allow the body to make the necessary physical, psychological and the neuro-endocrine adjustments required to cope with the demands of a homeostatic challenge.

The communication box method is a useful model to investigate the physiological changes that occur under psychological stress, since it can produce an experimental anxiety based on psychological communication between two or more animals, without the direct physical stress. In this animal model, the psychologically stressed rats are exposed to the visual, olfactory, auditory stimuli (such as struggling, vocalization, defecating, urinating and jumping) from the foot shock rat (Oishi *et al*., 2003). In the present study, we examined the neuro-endocrine and behavioural responses after different durations of inescapable foot shock and the subsequent effect of citalopram (10

milligram/kilogram, intraperitoneal once a day for 10 days), a selective serotonin reuptake inhibitor in reversing these responses. We have subjected rats to a number of stress paradigms (varying in duration), and assessed the effects thereof on behaviour at two different time points. Physically stressed rats were subjected to 10 unpredicted electric foot shocks (0.5 milliampere), in 10 minutes, while the psychologically stressed rats witnessed everything. The behavioural responses were assessed 5 days and 10 days after the last stress session. The rats were decapitated and corticosterone concentrations were determined one day after the open field and elevated plus-maze tests were performed.



The behavioural and endocrine responses in the rats subjected to physical and psychological stress in this study showed that single stress exposure may lead to different outcomes as repetitive stress exposure and that the consequences of stress exposure develop over time and persist for an extended time period. These consequences of direct stress exposure versus indirect stress exposure show a grading in stress intensity and perception, similar to that observed in humans. In the experiment where the rats where treated with citalopram, it showed that citalopram is effective in reversing anxious-like behaviours, but not locomotor deficits. In all the animals basal plasma corticosterone concentrations were comparable and physically and psychologically stressed rats displayed a hyposensitive hypothalamic-pituitary-adrenal axis following acute restraint stress.

These findings are interesting in a number of ways. It showed that our stress models propose to be useful in elucidating the complex interrelationship between an external event or stressor, and the organism experiencing it. Simultaneously it presents a promising platform for the finding of new or better treatments for patients

OPSOMMING

Spanning word beskou as een van die belangrikste faktore wat betrokke is by die patogenese van emosionele ongesteldheid, byvoorbeeld, direkte en indirekte blootstelling aan terroriste aanvalle of die onderwerping aan subtiele viktimisasie. Daar is ’n lang tydperk van prosedure ontwikkeling vir die bepaling van angstige gedrag in diere om sodoende nuwe en beter behandelings te ontwikkel vir pasiënte. Dit is bekend dat die blootstelling aan spanning voor die tyd, die daaropvolgende reaksie kan verander en dat die manier wat ‘n mens spanning hanteer ‘n invloed kan hê op jou gesondheid. Hierdie georkestreerde proses, bekend as die “spannings reaksie”, waar by verskeie meganismes betrokke is, laat die liggaam toe om die nodige fisiese, psigologiese en neuro-endokriene veranderings wat nodig is aan te bring, om al die homeostatiese uitdagings en eise te hanteer.

Die kommunikasie kas metode word gebruik om die fisiologies veranderings wat plaasvind tydens psigologiese spanning te ondersoek, aangesien dit eksperimentele angstigheid oplewer. Laasgenoemde word gebaseer op psigologiese kommunikasie tussen twee of meer diere, sonder direkte fisiese spanning. Die diere, in die een helfte van die kas, word blootgestel aan visuele, reuk en gehoor prikkeling of stimulus, van die dier in die ander helfte van die kas. Hierdie blootstelling staan bekend as psigologiese prikkeling van die rot (fisiese rot) wat elektries geskok word in sy pote. In die huidige studie

word neuro-endokrinologie en gedrag ondersoek na die blootstelling aan verskillende duurte van elektriese voetskokke asook die effek van citalopram (10 milligram/kilogram intraperitoneaal, een keer per dag vir 10 dae), ’n selektiewe serotonien heropname inhibeerder, op hierdie blootstellings. Rotte is onderwerp aan verskillende duurtes van spanningtoestande en die effek daarvan op gedrag, na 5 dae en na 10 dae is ondersoek. Die rot wat aan fisiese spanning blootgestel is, is onderwerp aan 10 onvoorspelbare elektriese voetskokke (0,5 milliampere) vir 10 minute, terwyl die rot wat aan psigologiese spanning blootgestel word, alles kan waarneem. Gedrag was bepaal 5 dae en 10 dae na die laaste voetskok sessie. Een dag na die bepaling van gedrag is die rotte gedekapiteer en kortikosteroon konsentrasies is bepaal.

Die endokriene- en gedragsdata verkry deur middel van fisiese en psigologiese spanning in hierdie studie beklemtoon die volgende: ‘n Enkele en herhaalde blootstelling aan spanning het nie dieselfde uitkoms nie en die gevolge van die bloostelling aan spanning ontwikkel oor ’n tydperk en kan voortduur vir ’n geruime tyd. Die gevolge van direkte versus indirekte bloostelling aan spanning wys, net soos in die mens, ’n gradering in spanningintensiteit en persepsie. In die eksperiment waar die rotte behandel is met citalorpam, is citalopram effektief in die omkering van angstige gedrag, maar oneffektief in lokomotoriese versteurings. In al die rotte was die basale plasma kortikosteroon konsentrasie vergelykbaar en in ’n akute spanningstoestand (gevolg van inperking), weerspieël beide die rotte wat aan fisiese en psigologiese spanning onderwerp,

’n hiposensitiewe hipotalmiese-pituitere-adrenale as. Hierdie data is interessant en dit bepaal dat die modelle wat gebruik is in hierdie studie, die komplekse verwantskap probeer verklaar tussen ’n eksterne gebeurtenis en die organisme wat dit waarneem of ondervind. Terselfdertyd, dien dit as basis vir beter en nuwe behandelings vir pasiënte.

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CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

Despite the fact that extensive research has been done investigating stress, a clear and scientifically accepted definition of stress does not yet exist. In 1936, Hans Selye believed stress is a necessary part of life and to cope with challenges is what life is all about. He defined stress as the non-specific response of the body to any demand imposed upon it. Later on, Selye himself tried to correct this one-sided over-generalization by distinguishing "eustress" from "distress". In 1929, Walter Cannon demonstrated in several seminal experiments that the sympatho-adrenal system was responsible for coordinating the "fight or flight" response necessary to meet external challenges. Cannon was able to show that both physical and psychological disturbances triggered the same response from the organism. He also proposed that there was a "critical" level of stress, in terms of magnitude and duration, against which the mechanisms fail and the organism perishes (as cited by Johnson *et al.,* 1992).

Organisms exist in a continually changing environment and the mechanisms that have evolved to cope with change, are organized within a nested hierarchy. The most conservative functions deal with homeostasis, the most ancient and urgent of needs (Greenberg *et al.,* 2002). According to Sapolsky (2000 b), what stressed most organisms is a "challenge to homeostasis". Homeostasis (Greek for "steady

state") is a term referring to "being in balance" and being knocked out of homeostatic balance causes stress. Greenberg *et al.* (2002) states that homeostasis is not an organism's only need and Bruce McEwen's succinct definition is an excellent beginning to a fuller appreciation of that fact: "Stress may be defined as a threat, real or implied, to the psychological or physiological integrity of an individual" (Greenberg *et al*., 2002; McEwen, 2000; Sapolsky, 2000 b).

Given the alterations in the central nervous system following stress exposure, numerous experiments have been done to elucidate the mechanism and relevance of this phenomenon in mood and anxiety disorders. The proportion of people (about one out of five) who experience depression or anxiety of clinical severity is astounding (Nesse, 1999). Elevated concentrations of cortisol, a steroid hormone used as an index of stress, are often observed in individuals suffering from these disorders, suggesting abnormalities in the regulation of the hypothalamic-pituitary-adrenal axis of these patients (Campbell *et al*., 2003).

A relatively new concept by Goldstein (2003), proposes that stress responses have a primitive kind of specificity, with differential responses of the sympathetic nervous and adrenomedullary hormonal systems, depending on the type and intensity of the stressor as sensed by the organism and interpreted in light of its experience. This suggests that stressors may be classified into different classes and these classes may evoke a differential response within the organism.

1.2. NEUROBIOLOGY OF STRESS

1.2.1. AN OVERVIEW OF THE STRESS RESPONSE

Stress is an adaptive response that is not unusual or unique only to certain individuals. In humans and animals, internal mechanisms allow the individual to maximize their chances of survival when confronted with a stressor. A stressor in this context is any situation that represents an actual or perceived threat to the homeostasis of the organism. Stress induces a coordinated biological, behavioural and psychological response (Mayer, 2001; Johnson *et al*., 1992).

This orchestrated process, usually referred to as the "stress response" (figure 1.1), involves various mechanisms, which allow the body to make the necessary physiological and metabolic adjustments required to cope with the demands of a homeostatic challenge. These adjustments may occur on the psychological (psychological and cognitive), behavioural (fight and flight), and biological level (altered autonomic and neuro-endocrine function). Among these responses, the neuro-endocrine limbic system consists of activation of the "fight or flight" sympathetic-adrenomedullary system, resulting in the release of catecholamines, as well as the activation of the hypothalamic-pituitary-adrenal axis (HPA-axis), which results in the release of glucocorticoids.

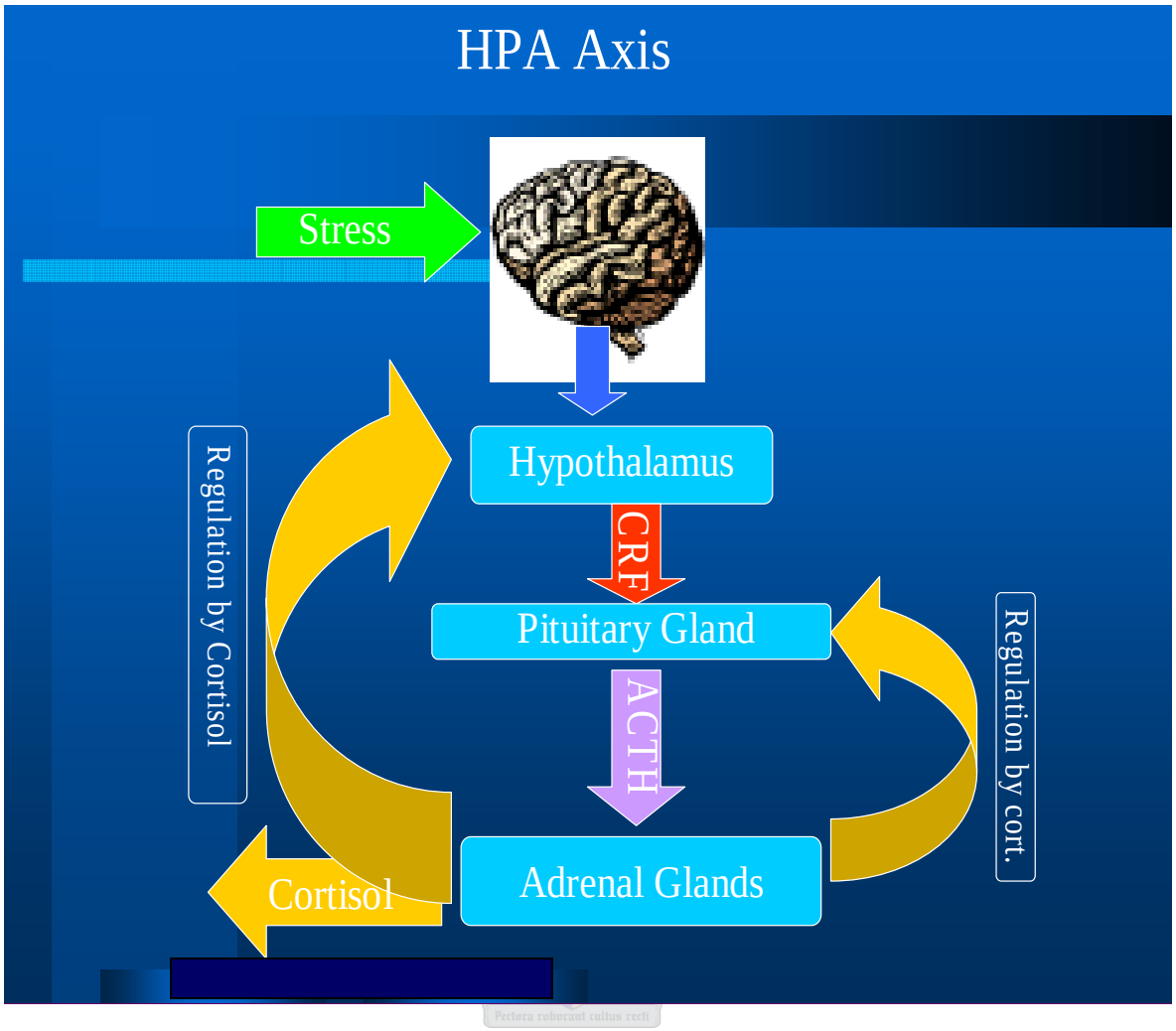


Figure 1.1: The Stress Response (Coker, 2000).

These primary hormonal mediators of the stress response are often referred to as the "stress hormones". In the short run, these hormones are essential for adaptation, homeostatic maintenance and survival, but when extended over longer time intervals, they exert a cost that can accelerate disease processes (Trentani, 2003; Mar Sanchez *et al*., 2001). Ultimately, Sapolsky (2000, a and b) stipulates that the stress response may become more damaging than the stressor itself. The adaptive response to stress appears to depend upon the quality (physical and

psychological), strength, and duration (acute or chronic) of the stimulus, as well as the constitution and state of the organism (Johnson *et al.,* 1992). These responses may interact with and be complemented by intrinsic rhythms and responses to chronic or intermittent stressors involving the hypothalamic-pituitary-adrenal axis. Varying patterns of responses to stressors are also affected by an animal's assessment of their prospects for successful coping. Subsequent central and systemic consequences of the stress response include apparent changes in affect, motivation and cognition that can result in an altered relationship to environmental and social stimuli (Greenberg *et al.,* 2002).

Stressors that challenge homeostasis, often regarded as the most urgent of needs, are the best known. When an organism's competence to maintain homeostasis within a specific range is exceeded, responses are evoked that enable the organism to cope by either removing the stressor or facilitating coexistence with it (Greenberg *et al.,* 2002; Yehuda and Antelman, 1993). While many stressors can evoke dramatic neural and endocrine responses, a more modest or 'sub clinical" response may be exhibited in response to milder stimuli.

In addition, stressors may be acute, sequential, episodic, chronically intermittent, sustained or anticipated, (Sapolsky, 2003). And whether considering childhood, old age, or any point in between, optimal function does not arise from a life without challenge. Instead, it involves the optimal amount of challenge, typically referred to as stimulation. Virtually by definition, what we view to be stimulatory is transient

exposure to a mild stressor. In contrast, a truly prolonged challenge, no matter how mild, is aversive. In this regard, that is why a roller coaster ride is 3 minutes, rather than 3 weeks in duration. The effects of mild, transient stressors enhance hippocampal-dependent cognition. However, more severe or prolonged stressors upon such cognition, forming an “inverse-U” pattern; the transition from sub physiological or basal glucocorticoid concentrations into the mild stress range enhances cognition, and elevations beyond that, impairs cognition (Sapolsky, 2003).

The physiological responses to stressors include cardiovascular, renal, visceral, cutaneous and metabolic changes in which catecholamines and hormones co-released from the sympatho-adrenal system, act as crucial mediators. Although the stress response enables the organism under attack to prepare itself quickly for fighting or escaping, prolonged or repeated stress is associated with a variety of disorders (Verago *et al.,* 2001). For example, repeated exposure to stressors can produce behavioural changes in a variety of learning tasks, which have been referred to as part of learned helplessness effects (Jodar *et al*., 1994). This phenomenon is frequently associated with depression (Seligman, 1992). Some cause alterations in central noradrenergic function that correlate significantly with depression. It is clear that exposure to different stressors results in stressor-specific response patterns and pathology. Different stressors are not necessarily interchangeable (Jedema, 2002). Electric foot shock has been reported to activate stress circuits involving alterations of neurotransmitter content in pre-synaptic

terminals as well as the active zone of the synapse influencing synaptic efficacy. Primary exposure to the foot shock stress served to activate adaptive mechanisms that restored the abnormal performance (Jodar *et al.,* 1994). This demonstrates that the brain has the capacity to establish different patterns of neural connection in response to the demands of the environment. However, in a study rats were exposed to a single 15-minute session of scrambled electric foot shocks (10 shocks of 6 seconds, 0.5 milliamperes). Two, six and ten weeks later, the rats were tested in the defensive withdrawal tests and the results were that a single session of foot shocks induced a long-term increase in defensive withdrawal behaviour, which persisted even after repeated testing for at least 10 weeks (Bruijnzeel *et al.*, 2001). These data showed that pre-shocked rats display long-term increased anxiety like behaviour in the defensive withdrawal test. A possible mechanism for this result may involve prolonged corticotropin-releasing hormone release, since a single session of foot shocks has been shown to increase the amount of corticotropin-releasing factor as well as vasopressin in corticotropin-releasing hormone terminals in the median eminence. These changes were evident for at least 11 days after the session (Schmidt *et al.*, 1996). It is evident from these experiments that duration to foot shock stress alone can give varied results, thereby reflecting the complexity of the stress response.

This complexity is borne out of the fact that the systemic aspect of stressors mainly affects brainstem or hypothalamic areas, while psychological stressors chiefly depend on processing in limbic areas. Interestingly, these different stress-

responsive brain areas all have direct or indirect projections to the paraventricular nucleus through which they influence the hypothalamic-pituitary-adrenal axis activity (Karssen, 2003).

1.2.2. THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

The neuro-endocrine system that regulates the secretion of glucocorticoids by the adrenal glands is known as the hypothalamic-pituitary-adrenal axis.

The region in the hypothalamus with the highest expression of corticotropin-releasing-hormone is the medial parvicellular part of the paraventricular nucleus (PVN). The paraventricular nucleus appears to sum and integrate input from numerous loci throughout the brain. The output of the paraventricular nucleus, release of corticotropin-releasing-hormone and arginine-vasopressin signifies the activation of the hypothalamic-pituitary-adrenal axis during stress.

Input to the paraventricular nucleus is divided into five classes, depending on the source of the input. Inputs come from:

1. brainstem pathways, which are mainly catecholaminergic, that appear to be involved in transmission of visceral information;
2. midbrain and pons cell groups that relay somatic and special sensory information;
3. the forebrain, from components traditionally classified as the “limbic system”, which is thought to mediate cognition and emotion;

4. circumventricular organs, which convey information from blood-borne chemosensory signals, and
5. the hypothalamus itself (figure 1.2), in connections that may provide information about an animal's motivational state or that may integrate stress-specific signals from other input classes (Zigmond *et al*., 1999).

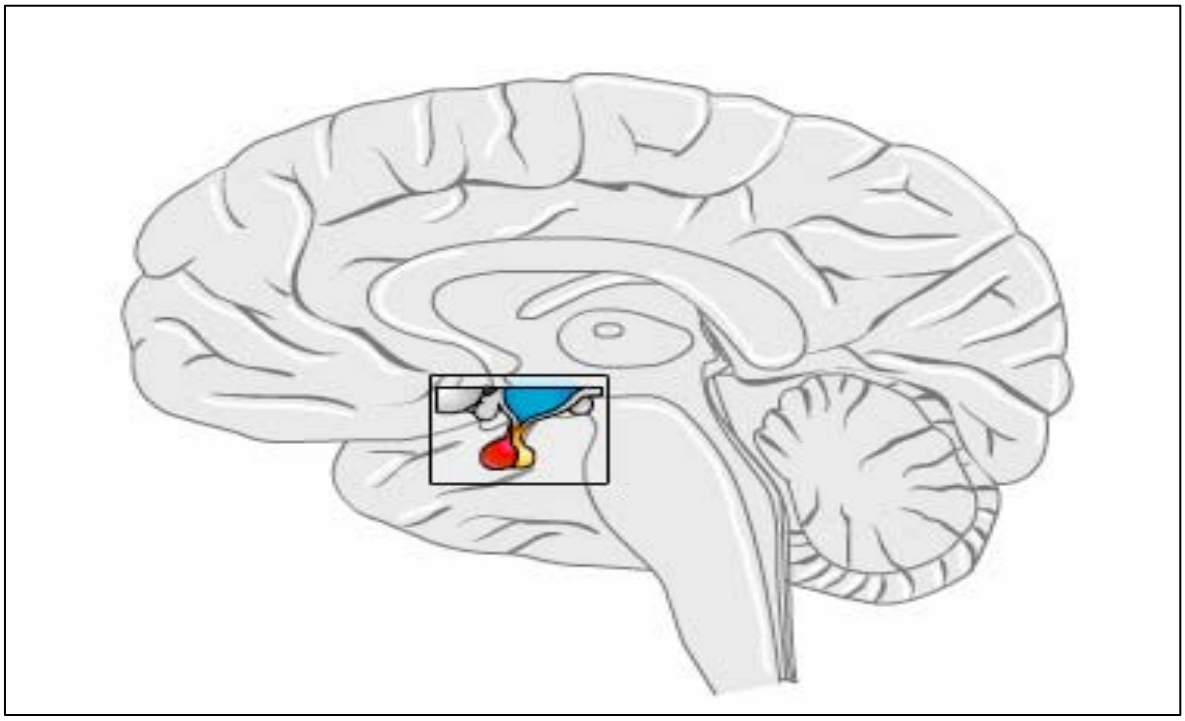


Figure 1.2: The hypothalamus triggers off the stress response (Driesen, 2004).

The hypothalamic-pituitary-adrenal axis meets the demands of stress primarily through the synthesis and release of 3 key hormones, such as corticotropin-releasing-hormone, adrenocorticotropic hormone, and the species-specific glucocorticoids, either cortisol (in human and non-human primate) or corticosterone (rodents) (Trentani, 2003). No major differences have been described so far

between the actions of corticosterone and cortisol. However, in most species, including humans, some corticosterone is also circulating e.g. in humans at 10 to 20 times lower levels than cortisol.

Upon activation by stress, neurosecretory neurons in the paraventricular nucleus of the hypothalamus secrete corticotropin-releasing-hormone into the portal circulation, through which they are transported to the anterior pituitary (figure 1.3).

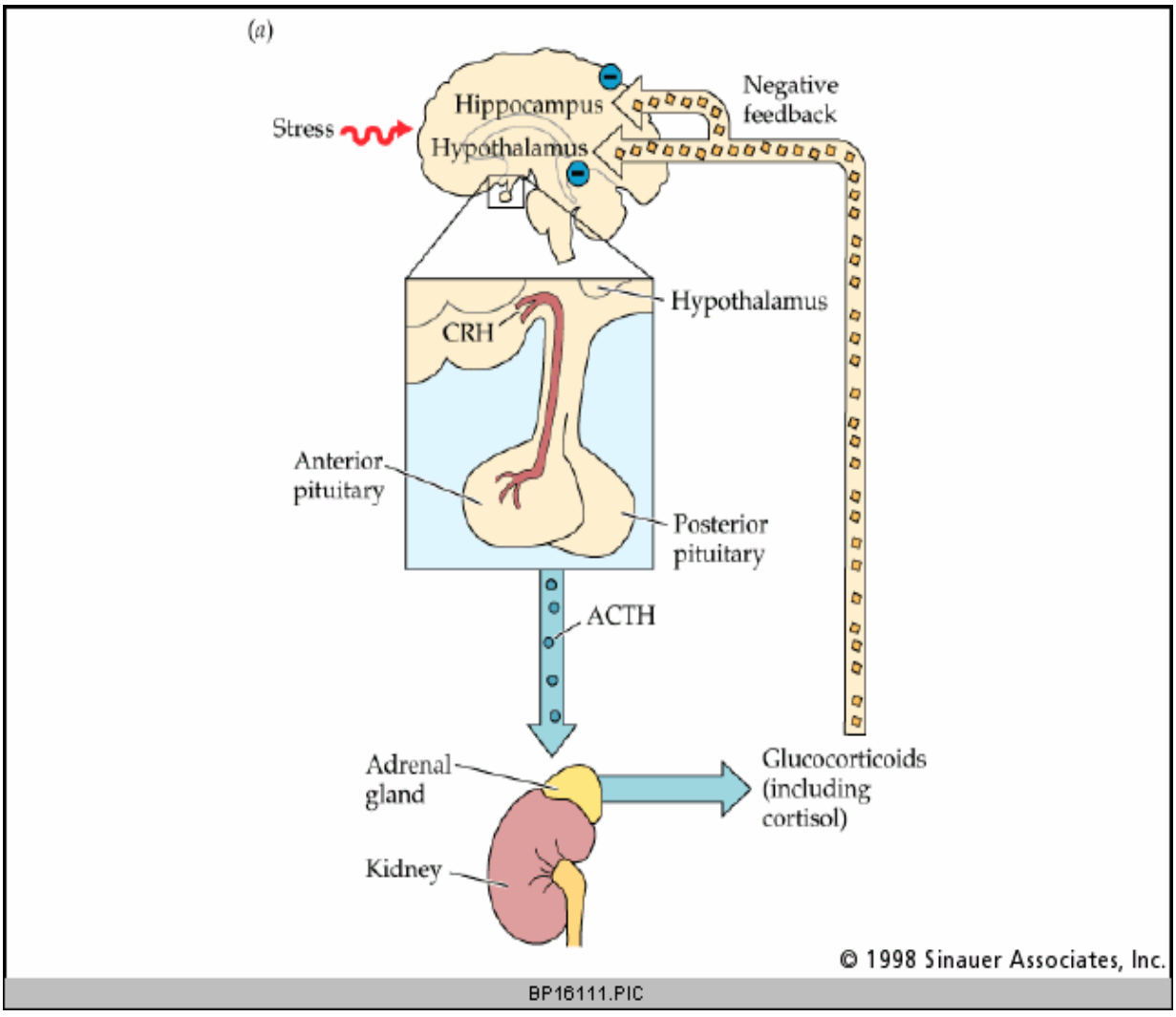


Figure 1.3: Outline of control of cortisol secretion. Adapted from Fraser, (1994).

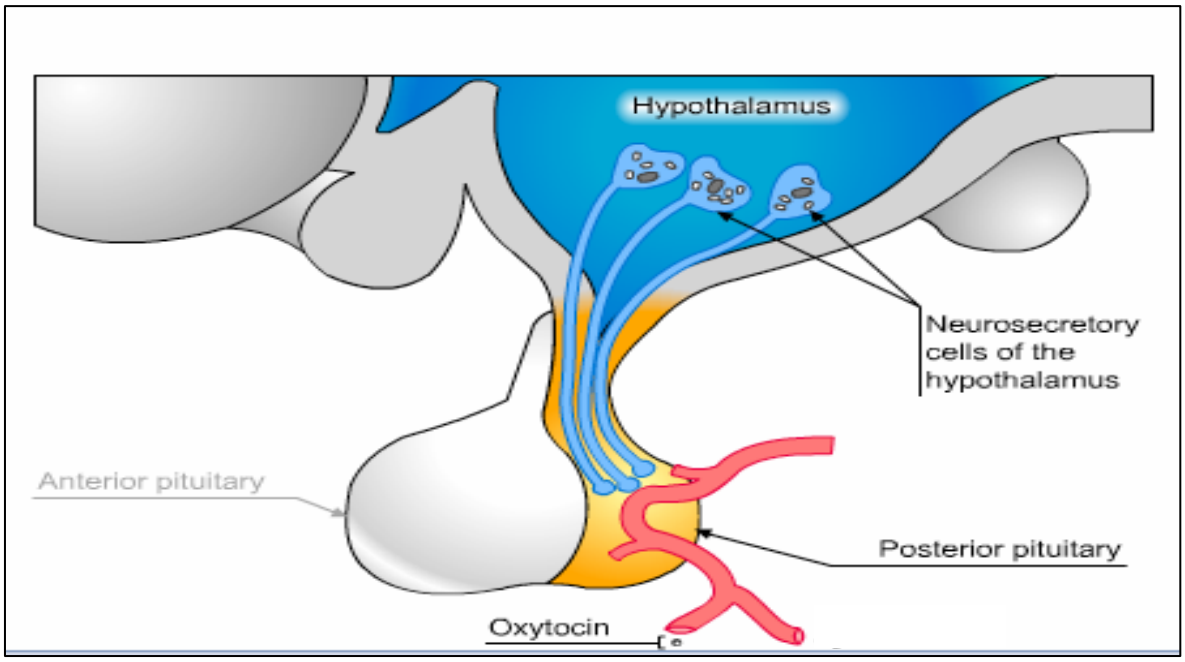


Figure 1.4: Posterior and anterior pituitary (Driesen, 2004).

In addition, corticotropin-releasing-hormone neurons express and release arginine-vasopressin, although most arginine-vasopressin is expressed in neighbouring magnocellular elements of the paraventricular nucleus that project to the posterior pituitary (figure 1.4). Corticotropes in the anterior pituitary express receptors for both corticotropin-releasing-hormones and arginine-vasopressin. Because they activate different signal transduction pathways, corticotropin-releasing-hormone and arginine-vasopressin act synergistically augmenting the release of adrenocorticotropin hormone from the anterior pituitary.

Subsequently, adrenocorticotropin hormone is transported by the systemic circulation to the adrenal glands where it interacts with adreno-cortical receptors, causing steroidogenesis and elevation of plasma glucocorticoids. Glucocorticoids

are then rapidly secreted into the bloodstream. Adrenal cortical cells do not store glucocorticoids at rest (Zigmond *et al*., 1999).

In non-stressful situations, both corticotropin-releasing-hormone and arginine-vasopressin are secreted in the portal system in a circadian, pulsatile fashion. During acute stress however, the amplitude and synchronization of corticotropin-releasing-hormone and arginine-vasopressin pulsations markedly increase, resulting in increases in adrenocorticotropin hormone and corticosteroid secretory episodes. Glucocorticoids are the final effectors of the hypothalamic-pituitary-adrenal axis and participate in the control of body homeostasis and response to stress (Trentani, 2003).

The brain-pituitary-adrenal axis is not simply an alarm system activated by stress. Instead, the axis exhibits basal secretion with daily oscillations. Basal glucocorticoid secretion follows a circadian pattern, with a peak at the start of the active period, which is in early morning for diurnal animals like humans and at onset of darkness for nocturnal animals like mice and rats.

The circadian rhythm (figure 1.5) is driven by the biological clock in the suprachiasmatic nucleus, which conveys excitatory and inhibitory activity to the paraventricular nucleus. Furthermore, the suprachiasmatic nucleus regulates adrenal sensitivity to ACTH and thus corticoid secretion directly via sympathetic neural input to the adrenal gland (Karssen, 2003).

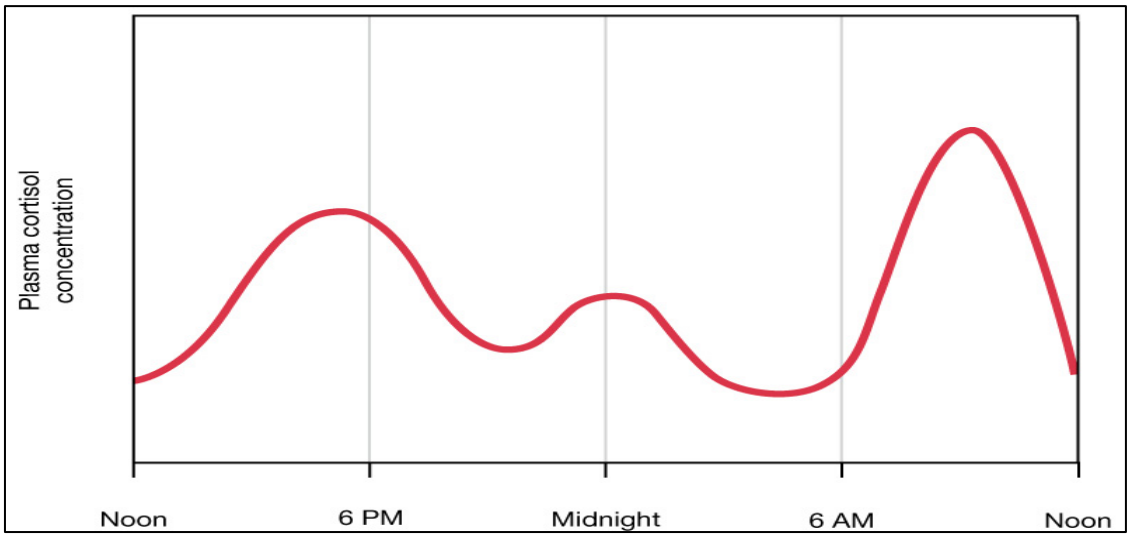


Figure 1.5: Human circadian rhythm (Booth, 2004).

Ottenweller (1992) studied the chronic stress state in rats and determined the number of stressor exposures necessary to induce a chronic stress state. The rats were given two hours of inescapable shock on three, four, seven and ten consecutive days. The data suggest that 3 days of the stress regimen are sufficient to produce a state of chronic stress as more exposure to stressors did not produce higher corticosterone levels or greater behavioural changes.

Repeated exposure to a stressor of a chronic stress state caused elevated plasma corticosterone levels in the morning while the evening levels were similar in stressed rats and controls (Ottenweller, 1994). This may be because the hypothalamic-pituitary-adrenal axis is more sensitive to glucocorticoids feedback in the morning and that the mechanism mediating feedback in the evenings may be disrupted by repeated stressor exposure. Ishikawa *et al*. (1992) showed in an acute stress experiment, that in psychological (non-foot shocked) and foot shocked

rats, during the light phase (09h00 – 15h00) the induced increased plasma corticosterone levels return to corresponding control levels six hours following the initiation of the stress session, while those in the dark phase (21h00 – 03h00) remained significantly higher.

The response of the brain to both acute and chronic stress must be regarded in terms of its capacity to show adaptive plasticity. The stress-induced remodeling of hippocampal dendrites and suppression of neurogenesis may serve as a protective function in the short run against serious excitotoxic damage, and it also appears to go hand-in-hand with the adjustments of the animal's behaviour to a long-term change in the social and physical environment. For these changes, circulating glucocorticoids and catecholamines act together with excitatory amino acids, serotonin and other neurotransmitters (McEwen, 2000).

1.2.3. GLUCOCORTICOIDS

Glucocorticoids are the adrenal steroid hormones secreted in response to stress. The hormones are central to successfully coping with a major physical stressor (such as fleeing a predator), as they mobilize stored energy, increase cardiovascular tone and suppress costly anabolism, such as growth, tissue repair, reproduction, digestion and immunity, for more auspicious times (Sapolsky, 2003). Adrenal corticosteroids are therefore essential for life, as they coordinate the responses of body and brain to changes in both the external and internal environment. They play a crucial role in homeostasis, which comprises the

processes of maintaining the internal environment of the body in a condition consistent with survival of the individual (Karssen, 2003).

Generally two subgroups of adrenal corticosteroids are recognized, mineralocorticoids and glucocorticoids (Karssen, 2003). Mineralocorticoids and glucocorticoids differ in their distribution and in their affinities for corticosteroid receptors. The glucocorticoid receptors are localized throughout the body, including thymus, anterior pituitary and brain. Particularly high densities are found in the parvocellular neurons of the paraventricular nucleus, where glucocorticoids regulate the expression of corticotropin-releasing hormone and arginine-vasopressin, and in neurons of the hippocampal formation, which is a brain region involved in regulation of the behavioural stress response. The mineralocorticoid receptors are mainly localized in the kidneys and in limbic regions of the brain, such as hippocampus, septum and amygdala. Mineralocorticoid receptors are almost fully occupied at low levels of circulating corticosterone, whereas glucocorticoids receptors only become saturated at higher levels of corticosterone, seen after stress and at the diurnal peak. Upon activation, these mineralocorticoids and glucocorticoids will migrate into the nucleus, where they act as transcription factors and reduce or induce the transcription of genes. Dysregulation of corticosteroid homeostasis, seen after chronic stress, disrupts the mineralocorticoid and glucocorticoid balance in the brain. This changed balance disrupts the serotonin neurotransmission through a dominance of mineralocorticoid mediated suppressive effects on the raphe-hippocampal system. For instance, chronic release of high

corticosterone levels decreases serotonin –1A receptor binding in the dorsal hippocampus (Veenema *et al.,* 2003).

Termination of the stress response by adrenal steroids appears to operate through three different and partially independent mechanisms: a rate sensitive fast feedback, an intermediate feedback, and a delayed feedback mechanism. The fast feedback mechanism is a very rapid phenomenon (with a time domain of 5-15 minutes), activated by the rate of rise of plasma glucocorticoid levels rather than their absolute concentration. This inhibition is achieved by glucocorticoids binding to specific receptors in selective limbic regions including the hypothalamus and the hippocampus. Intermediate and delayed-feedbacks operate relatively slowly over the course of hours to days and are activated by the interaction of the glucocorticoid-receptor complex with genes in the hypothalamus. The result of this interaction is the suppression of the expression of selective genes, thereby decreasing the secretory drive in the pituitary. These direct feedback mechanisms however cannot account for all aspects of hypothalamic-pituitary-adrenal axis inhibition supporting the hypothesis of the existence of other neural inhibitory pathways working in parallel with steroid feedback. Thus, although direct glucocorticoid inhibitory action accounts in part for the ability of maintaining the organism in an ideal state of stress responsiveness, neural connections from the hippocampus and prefrontal cortex to the hypothalamus also play a critical role in the regulation of hypothalamic-pituitary-adrenal axis response to stress (Trentani, 2003).

Sapolsky (2003) postulates that the permissive actions controlling the sensitivity of the stress system are evident at low levels of glucocorticoids, whereas the suppressive or stimulating actions facilitating the adaptation to stress, emerge only when glucocorticoid levels are raised as following the stressor (Karssen, 2003). Glucocorticoids prevent primary stress responses from overreaction, thus protecting the individual against the potential threat to homeostasis caused by its own defense systems.

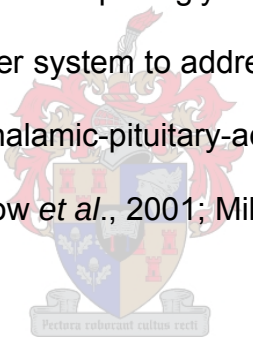
1.2.4. NEUROTRANSMITTERS INVOLVED IN THE STRESS RESPONSE

Modulation of the stress response involves the coordinated activity of multiple systems to allow regulation through interconnections at various levels. The hypothalamic-pituitary-adrenal axis has important functional interactions with the central norepinephrine system. The corticotropin-releasing-hormone/norepinephrine system serves as a generalized warning structure to help determine whether, under threat, an individual's attention should turn towards external sensory stimuli or to internal vegetative states. In conjunction with the autonomic nervous system, the corticotropin-releasing-hormone/norepinephrine system plays an important role in the maintenance of homeostasis following exposure to stressors. Noradrenergic activation stimulates corticotropin-releasing-hormone release, and in the central amygdala, this neuropeptide in turn, may itself modulate certain behavioural responses to stress. This nucleus is consistently involved in the organization of processes of passive coping, reflected by immobile behaviour and parasympathetic activity (Trentani, 2003; Baez *et al*., 1996; Johnson

et al., 1992). Besides norepinephrine, other neurotransmitter systems have also been implicated in the modulation of the stress response, including the serotonergic system.

1.2.4.1. SEROTONIN

Serotonin is involved in the regulation of a variety of different processes, including fear, anxiety, arousal, aggression, mood, impulsivity, and food-intake regulation (Trentani, 2003). A number of studies has shown the existence of an interaction between glucocorticoids and the serotonin system (De Kloet, 2003; Helmeke *et al*., 2001; Liberzon *et al.,* 1997). Not surprisingly therefore that clinical studies have focussed on this neurotransmitter system to address symptoms that are associated with abnormalities in the hypothalamic-pituitary-adrenal axis (Pariante *et al*., 2004; Tjurmina *et al.,* 2004*;* Attenburrow *et al*., 2001; Millan *et al*., 1998)



1.2.4.2. SELECTIVE SEROTONIN RECEPTOR INHIBITORS IN PSYCHIATRIC DISORDERS

Malfunctioning of the serotonergic system and dysregulation of the hypothalamic-pituitary-adrenal axis has been implicated in the pathophysiology of depression and other affective disorders. In the human brain, serotonin containing neurons are highly localized in specific clusters in the brainstem and spinal cord. Axons from these cells end in serotonin containing terminals innervating diverse areas throughout the brain. Given these distributions, dysfunction of serotonin neurons has been implicated in various diseases, including major depression, anxiety

disorders, obsessive-compulsive behaviour and post-traumatic-stress disorder (Stengler-Wenzke *et al.,* 2004; Vaswani, *et al*., 2003).

The serotonin transporter (5-HTT) is known as the site of action of the serotonin reuptake inhibitors, which were initially developed as antidepressants, but now are the most widely used agents in the treatment of many additional neuropsychiatric and related disorders. It was discovered that the gene that expresses the serotonin transporter possesses a functional promoter-region polymorphism, which is associated with temperament and personality traits such as anxiety and negative affective states (Murphy *et al*., 2001).

Therapeutic response to selective serotonin reuptake inhibitors and the absence of improvement with norepinephrine reuptake inhibitors and dopamine agonists argue strongly for a role of serotonin in the pathophysiology and treatment of obsessive-compulsive disorder. Stengler-Wenzke *et al.* did a study in 2004 on ten age-related obsessive-compulsive behaviour patients and found a significant reduction in serotonin availability in the midbrain and upper brainstem. This may result from a reduced number of serotonergic (raphe) neurons that because of genetic predisposition are prone to degeneration.

Patients with generalized anxiety disorders experience chronic excessive uncontrollable worry combined with irritability, sleep disturbance and muscle

tension. The role of serotonin in the mediation of these anxiety symptoms is obviously complex.

1.2.4.3. MECHANISM OF ACTION OF SELECTIVE SEROTONIN INHIBITORS

Selective-serotonin-reuptake-inhibitors are selective in terms of affecting the neuronal uptake transporter for serotonin and they affect a multitude of specific post synaptic serotonin receptors (figure 1.6). Of the many serotonin receptor subtypes, only a few have been characterized with respect to their role in neuro-endocrine functions. For example, the serotonin-1A and -2A receptors are involved in adrenocorticotropin release, while -1A, -2A and –2C have been shown to affect oxytocin secretion. Arginine-vasopressin release may be mediated by –2C receptors. By virtue of their ability to inhibit the reuptake of serotonin, the selective-serotonin-reuptake-inhibitors elevate the extracellular levels of serotonin in the synapse. Consequently, the activation of the post-synaptic serotonin receptors in the hypothalamus is improved, leading to the secretion of several hormones (Raap and Van de Kar, 1999).

Acute administration of selective-serotonin-reuptake-inhibitors, block serotonin reuptake in the cell body region in the raphe, subsequently activating somatodendritic serotonin-1A receptors. These serotonin-1A autoreceptors in the raphe nuclei provide negative feedback inhibition of serotonergic firing. Therefore, the tendency of these drugs to increase the levels of serotonin in the synapse is often negated by activation of the negative feedback, leading to reduced release of

serotonin in the forebrain. Less activation of the post-synaptic serotonin receptors occurs and a less robust neuro-endocrine response to acute administration of selective-serotonin-reuptake-inhibitors is observed (Raap and Van de Kar, 1999).

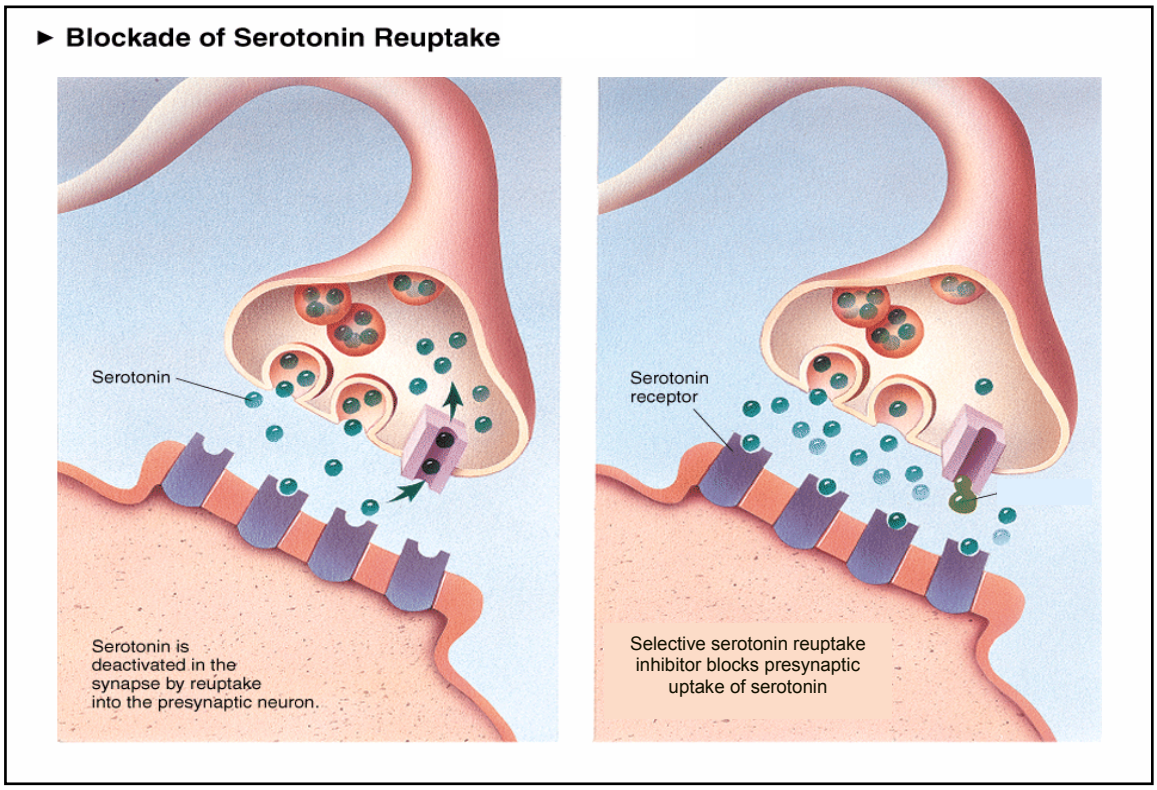
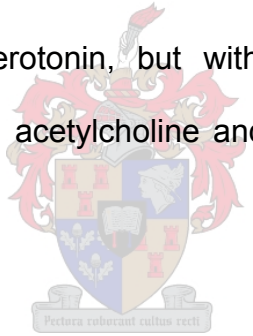


Figure 1.6: Mechanism of selective serotonin inhibitors. Selective serotonin inhibitors block the uptake of serotonin, thus increasing the activation of serotonin receptors (Driesen, 2004).

In general, with long-term exposure to selective-serotonin-reuptake-inhibitors, no consistent alterations in basal levels of any hormone were observed in studies done in rats and human volunteers (Pariante *et al.*, 2004; Jensen *et al.*, 1999; Raap and Van de Kar, 1999; Johnson *et al.*, 1992). Serotonin concentrations are

increased in the synaptic cleft by these inhibitors and most studies have focused on post-synaptic serotonin-1A, -2A or-2C receptor systems. Because these serotonin receptors are linked through G-proteins to second messenger enzymes, activation of each receptor leads to the secretion of multiple molecules of oxytocin or corticotrophin. During exposure to selective-serotonin-reuptake-inhibitors, no change was observed in the density of serotonin 1A receptor, but a decrease in the levels of the G-protein occurs (Makara and Haller, 2001; Raap and Van de Kar, 1999). This means that chronic exposure to selective-serotonin-reuptake-inhibitor reduces the coupling of serotonin-1A receptors to their effector's mechanisms. Selective-serotonin-reuptake-inhibitors were developed for inhibition of the neuronal uptake pump for serotonin, but without affecting the various other neuroreceptors (e.g. histamine, acetylcholine and adrenergic receptors) (Vaswani *et al.,* 2003).



Many serotonergic drugs are not effective after acute administration, which suggest that the primary mechanism of action is not antagonism of serotonin reuptake. The immediate actions of selective-serotonin-reuptake-inhibitors are mostly side effects that may be mediated by negative allosteric modulation of the serotonin transporter. Davis *et al.* (1999) postulated that serotonin modulates homeostasis between dopamine, noradrenaline and GABA, which mediate thought processes e.g. mood and anxiety. When this homeostasis is disturbed, psychiatric disorders set in and the serotonergic drug merely reinstates the homeostasis.

Ohi *et al.* (1989) performed a study on serotonin neuronal systems and stress adaptation after repeated foot shock for up to 10 days. Hypolocomotion, freezing behaviour and weight loss was observed after the initial stress, but relief from these behavioural changes developed by the third day and persisted for another 7 days, indicating the development of stress adaptation. Ohi *et al.* (1989) suggested that the hypersensitivity of the serotonergic system after repeated stress might be in part responsible for the maintenance of the stress adaptation. Inoue *et al.* (1994) examined the regional patterns of brain dopamine and serotonin activation after physical and psychological stress. Rats that were repeatedly exposed to 2.5mA foot shock for a period of 10 days displayed a greater degree of freezing-induced conditioned fear stress than those given only one foot shock session, indicating an augmentation of fear and stress intensity. Conditioned fear stress, after repeated foot shock, increased the dopamine metabolism in most of the brain regions except the striatum and increased serotonin metabolism in the medial prefrontal cortex, nucleus accumbens and amygdala. These results suggest that regional patterns of brain dopamine and serotonin activation after physical and psychological stress depend on the intensity of the stress. It also suggests that the wide spread activation of these two neurotransmitter systems, after more severe stress, might relate to behavioural changes that reflect the augmentation of fear.

In another study, Shalom *et al.* (2003) found that serotonin levels in the cortex and hypothalamus were not affected after 7 days of administration of 5 milligram/kilogram fluoxetine (a selective serotonin re-uptake inhibitor), but were

increased in the cortex after 12 days of administration of the same drug. This result showed that varying the duration of treatment might yield totally different outcomes.

1.2.4.4. PHARMACOLOGY OF A SELECTIVE SEROTONIN REUPTAKE INHIBITOR - CITALOPRAM

Selective serotonin reuptake inhibitors are the most prescribed antidepressant drugs for the treatment of depression. The mechanism of action of the SSRI, escitalopram (Citalopram) is linked to the potentiation of serotonergic activation in the central nervous system, resulting from inhibition of neuronal reuptake of serotonin. Citalopram has a high selectivity for inhibiting serotonin reuptake and this action is primarily due to the action of the (S)-enantiomer. In addition, it has some affinity to α-1-adreno-receptors and slight histamine H_1 receptor blocking potency (Owen *et al*., 2001).

It is well established that the maximal therapeutic effect of selective serotonin reuptake inhibitors, like citalopram, are achieved after several weeks of treatment in for example depressive patients. It has been shown that hyperactivity in the hypothalamic-pituitary-adrenal axis is affected by long term anti-depressant treatment in association with the mood normalizing effect.

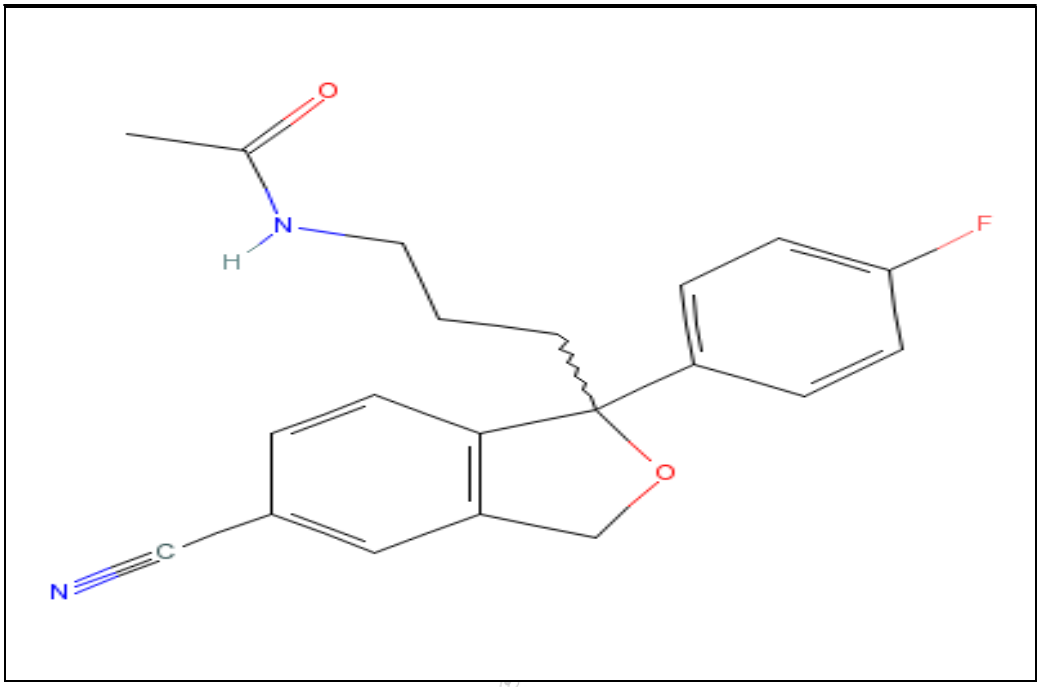


Figure 1.7: Structural formula of Citalopram (NIST Chemistry Web book)

Jensen *et al.* (1999) compared the acute (single injection of 10 milligram/kilogram) and the long term effects of citalopram (10 milligram/kilogram for 14 days) on the hypothalamic-pituitary-adrenal axis. A single injection of citalopram increased the plasma levels of adrenocorticotropin hormone within 30 minutes. This increase was associated with an elevation in the number of c-Fos containing cells in the paraventricular nucleus. On the other hand daily treatment for 14 days decreased the expression of proopiomelanocortin mRNA and the corticotropin-releasing-hormone-induced c-AMP accumulation in the pituitary, was also altered. The hypothalamic-pituitary-adrenal axis response was lower (in chronically treated animals) than after a single dose of citalopram. This suggests that the hypothalamic-pituitary-adrenal axis was desensitized by the repeated

administration of the citalopram. The mechanism of this desensitization remains unclear.

1.2.5. ABNORMAL STRESS AND DISEASES

It has been proposed that a critical factor in the pathophysiology of several psychiatric syndromes, such as major depression, anorexia nervosa and panic disorder, stems from an abnormality in the counter regulation of the generalized stress response, resulting in corticotropin-releasing-hormone and/or central catecholamine hypersecretion (Pariante *et al*., 2004; Newport and Nemeroff, 2000; Van de Kar and Blair, 1999; Johnson *et al.*, 1992). Sapolsky (2003) has proposed the glucocorticoid cascade hypothesis that may be of relevance to stress-induced disorders like depression. This model describes the effects of chronic stress on hippocampal neurons and states that chronic glucocorticoid administration down-regulates hippocampal steroid receptors, but not hypothalamic or pituitary receptors.

Animals with down-regulated hippocampal glucocorticoid receptors exhibit delays in the turnoff of the glucocorticoid response to stress and demonstrate decreased sensitivity to glucocorticoid fast feedback. This decrease in glucocorticoid receptors and insensitivity to negative feedback is thought to lead to prolonged hypersecretion (Kuipers, 2004). Other studies support this view that the negative feedback system within the hypothalamic-pituitary-adrenal axis is dysfunctional during depression (Newport and Nemeroff, 2000).

Post-traumatic stress disorder is viewed as an extreme form of stress-induced sensitization. Kandel and Schwartz (1982) defined sensitization as an elementary form of non-associative learning in which an animal learns to strengthen its defensive reflexes and to respond vigorously to a variety of previously neutral or indifferent stimuli after it has been exposed to a potentially threatening stimulus. The neurobiology of post-traumatic stress disorder bears striking similarities to that of major depression, however there are differences that underscore the uniqueness of each disorder as stress-induced syndrome. Both depression and post-traumatic disorder are associated with hyperactivity of the hypothalamic-pituitary-adrenal axis and the catecholamine/sympathetic nervous system, but post traumatic disorder are associated with normal to low cortisol levels, despite hypersecretion of corticotropin-releasing hormone. This and increased density of glucocorticoid receptors on peripheral lymphocytes suggest that hypocortisolemia in this disorder may be a consequence of exaggerated hypothalamic-pituitary-adrenal axis negative feedback (Newport and Nemeroff, 2000).

Liberzon *et al*., (1999) measured post-stress changes in hippocampal glucocorticoids receptor messenger RNA expression in acutely stressed rats. He demonstrated increased hippocampal glucocorticoids receptor messenger RNA expression but decreased hippocampal mineralocorticoid receptor messenger RNA expression (Liberzon *et al*., 1999). According to Newport and Nemeroff (2000), it remains unclear which of the neurobiological alterations observed in post-traumatic stress disorder thus far are a direct consequence of the disorder itself and which

are a consequence of homeostatic adaptations to trauma exposure independent of any illness.

1.2.6. PHYSICAL AND PSYCHOLOGICAL STRESS

Stress experiences can have both physical and psychological aversive components. However, experiments to investigate neurobiological differences between these two have been limited. Non-physical stress may cause fear, resulting from real and immediate danger, or anxiety, caused by uncertainty and imaginary threats (Pijlman, 2001). One of the tools used to study differences between physical and psychological stress is the communication box. This apparatus consists of two compartments. An animal is placed in each compartment, the one being exposed to a physical stress (electric foot shock is commonly used for this purpose), while the other witnesses the application of the physical stress. Using this experimental paradigm it was shown that in the acute stress procedure, plasma corticosterone levels were elevated during electric foot shock, but not the psychological stressed rats (Ishikawa *et al.*, 1992). In support of these data, Sato *et al.* (1996) reported that long-term physical stress exposure, but not the psychological stress, decreases catecholamine levels in the hypothalamus. The results of these studies (i.e. lack of corticosterone and catecholamine responses) may also be interpreted as evidence for questioning the existence of psychological stress in rats.

However in a recent study, although not convincing, Pijlman and Van Ree (2002) observed differences in the behaviours evoked by both physical and psychological stress respectively, when compared to controls. Physically stressed animals showed initial immobility in the large open field and the shock prod bury task, while the psychologically stressed animals displayed an increase in locomotor activity and exploration. Interestingly these behaviours disappeared over time, which led to the conclusion that neither physical stress nor psychological stress affects the coping style and anxiety levels of the rats. In similar studies psychological stress was achieved by exposing a witness rat to visual, auditory and olfactory information from another rat undergoing repeated foot shocks or exposure to high temperatures using hot plates (Takahashi *et al*., 1988; Ramsey and Van Ree, 1993 and Kuzmin *et al*., 1996). In these experiments psychological, but not physical stimulation (hot plates or foot shock) enhanced the initiation of intravenous self-administration of morphine and cocaine in rats and mice, respectively.

Therefore, it appears as if physical and psychological stresses may have different modalities and hence may be perceived as independent entities. Additional evidence comes from Helmeke *et al.* (2001), who studied the effect of physical and psychological stress during repeated parental separation. These authors found that psychological and physical stress altered synaptic development in the anterior “psychological” cingulate cortex differently: Psychological stress elevated spine densities, whereas physical stress (daily saline injections) reduced spine densities. Similarly, early social-psychological environment interfered with synaptic

development in the prefrontal cortex and other limbic areas. These experience-induced morphological changes may be the underlying reason for altered behavioural and cognitive capacities in later life, since the limbic system is critical for a variety of psychological behaviours and associative aspects of learning (Helmeke *et al*., 2001).

These studies suggest that a rat exposed to foot shock emits information that causes the witness rat to be subjected to a stressful situation as well. This psychological stress results in responses that are different from foot shock in terms of morphology and physiology (Helmeke *et al*., 2001; Ishikawa *et al.*, 1992).

1.2.6. ANIMAL MODELS TO STUDY STRESS RELATED DISORDERS

Animal models of human processes and disorders are useful for several reasons. First, they offer the possibility of simulating a human condition under controlled circumstances, with large numbers of subjects, in a simpler more readily understandable system. Second, in contrast to human disorders, which can be studied only after they become clinically manifest, animal models are observable as they evolve, permitting the study of symptoms as they developed. Third, they allow the testing of pharmacological and other prospective treatments that might be difficult or unethical in humans.

Animal models of stress and anxiety disorder have been useful in elucidating links between behavioural symptoms and biological abnormalities and in suggesting

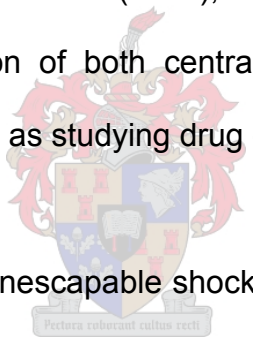
possible treatment strategies for the disease. These models have the potential to provide information about the course and etiology of stress disorders (Uys *et al*., 2003; Yehuda and Antelman, 1993).

Several experimental models have been used to elucidate stress mechanisms, including novelty, water immersion, immobilization, swimming and foot shock. (Verago *et al.*, 2001). Some of the currently available animal models of anxiety require measurement of physiological or endocrine functions, but the vast majority involves the study of behaviour. These behavioural models involve exposure of animals to stimuli that appear capable of causing anxiety in humans. They may be grouped into two general categories involving either conditioned or unconditioned responses. Conditioning models require considerable training of subjects, food or water deprivation, or the use of electric shock as an aversive stimulus. The study of unconditioned responses represent a logical extension of laboratory methods and provide a high degree of ecological validity for the research and a more complete characterization of the effects of experimental manipulations (Rodgers and Dalvi, 1997).

The communication box method is a useful model to investigate the physiological changes that occur under psychological stress, since it can produce an experimental anxiety based on psychological communication between two or more animals, without the direct physical stress. In this animal model, the psychologically stressed rats are exposed to the visual, olfactory, auditory stimuli

(such as struggling, vocalization, defecating, urinating and jumping) from the foot shock rat (Oishi *et al*., 2003).

The major use of restraint or immobilization has been as a “stressor” for the induction of the stress response. Restraint stress was applied by placing the animal inside a 25 x 7-cm plexiglass cylindrical restrainer (Song *et al.*, 2002), and minimizing movement by adjusting the tailgate of the apparatus. This restricted the ability of the animal to move. Holes were present for ventilation. The animals were subjected to this procedure for 10 minutes and then decapitated (Silveira *et al*., 2000). According to Pare and Glavin (1986), this technique has proven to be a very useful for the examination of both central and peripheral mechanisms of stress-related disorders, as well as studying drug effects upon these disorders.



Uncontrollable stress such as “inescapable shock” describes a specific behavioural paradigm in which animals are tested with a series of several shocks from which they cannot escape. After a brief exposure to trauma, an organism does not have the opportunity to “learn” a maladaptive response or learn to obtain “controllability” over its environment. The types of stimuli that induce anxiety behaviour can be over long before an individual can develop control, or helplessness, over the situation (Yehuda and Antelman, 1993).

Time-dependent sensitization refers to the fact that one exposure to a stressor (e.g. injection of pharmacologic agent or immobilization stress) can induce an extremely

long-lasting alteration in the subsequent responsiveness of the organism to pharmacological or non-pharmacological stressors. (Yehuda and Antelman, 1993). Ramos and Mormede (1998) stated that “locomotion in novel stressful environments and measures of anxiety represent two different dimensions of psychologicality”. They said that neuro-endocrine and autonomic parameters are adequate indicators of stress levels, whereas locomotor-activity can be influenced independently.

It has been established in literature of animal studies that different types of stress paradigms lead to different biobehavioural consequences and that many different factors contribute to differential responsivity to stress. Differential responsivity to stress can be influenced by factors other than the actual stressor, such as the state of the organism during stress, past stress history of the organism and even genetic makeup (Yehuda and Antelman, 1993). Therefore, it becomes important to differentiate between factors that are essential for the induction of stress-like symptoms and those that influence their manifestations.

The open-field task assesses the exploratory behaviour in a novel environment. The latency to explore, number of squares traversed, rearing and grooming frequency and centre square traversed are usually quantified to obtain insight into the level of anxiety in animals (Campbell *et al*., 2003).

It is widely accepted that repeated exposure to a stressful situation leads to a gradual reduction in behavioural, hormonal and cardiovascular responses, a process usually referred to as habituation. According to Van den Buuse *et al.* (2001), behavioural responses are reduced upon repeated exposure to the open field, in accordance with this principle. They also observed that rats, when exposed to the novelty stress of being placed in a large open field, they displayed marked pressor responses and tachycardia in addition to a range of behavioural manifestations. The behavioural hyperactivity in the open field is reduced upon repeated exposure, but interestingly the cardiovascular responses do not show similar habituation. According to de Boer *et al.* (1990), frequency of stressor presentation affects the adaptation pattern of neuro-endocrine and metabolic response to chronic intermittent stress. Neuro-endocrine adaptation to stress is therefore similar to the process of behavioural or neurophysiological habituation to a sensory stimulus (De Boer *et al*., 1990). Habituation will occur when stressors are predictable in terms of the type of stimulus, intensity and duration. This will allow the animal to reduce its behavioural and neurohormonal responses to the minimum required to maintain homeostasis (Van den Buuse *et al.,* 2001).

The elevated-plus maze test is probably the most popular of all currently available animal models of anxiety and is an excellent example of a model based on the study of behaviour (Rodgers and Dalvi, 1997). Montgomery (1955) reported that rats show consistently high levels of exploration of preferable enclosed alleys and concluded that, as open and enclosed alleys would evoke the same exploratory

drive, the avoidance of open alleys must be due to higher levels of fear. This model has subsequently been validated in our laboratory by Richter *et al.* (2003) and Pietersen *et al*. (2001).

The basic parameters recorded in this model comprise total open arm and total closed arm entries; time spent on the open arms and time spent in the closed arms; and total rearing and grooming. Normal exploratory behaviour is in favor of the closed arms and this tendency to stay in the closed arms can be enhanced by compounds that increase the aversion towards the anxiety-provoking open arms, e.g. anxiogenics. According to Rodgers and Dalvi (1997) rodents not only avoid the open arms of an elevate plus-maze, but also exhibit distinct behavioural and physiological indices of fear in this paradigm.

The elevated plus-maze has been extensively used in the study of mechanisms of anxiety and in routine drug screening. Given the queries about the utility of the test as a model of anxiety, the plus-maze is subject to extremely wide methodological variation between laboratories. Because of this, the validility of the elevated plus-maze as an animal model of anxiety was examined (Hogg, 1996). The following cautionary actions were highlighted:

1. Repeated handling of animals for several days before experimentation serves to habituate them to the stresses to which they are commonly subjected immediately before plus-maze testing;

2. The exposure of animals to the open field or holeboard apparatus before testing on the elevated plus-maze was suggested as a method to increase the general exploration of the maze, and in particular, that of the open arms.
3. Acute stressors (e.g. electrical shock, forced swimming, saline injection) have been reported to be influential on the behaviour exhibited by animals on the maze. All these enhance anxiety, similar to immobilization, social defeat and exposure to a cat reduce the exploration of the open arms.
4. Contradictory evidence on the effects of repeated testing on the elevate plus-maze has emerged. For instance a greater percentage of rats spent more time in the open arms and less time in the closed arms on the second exposure to the plus-maze, as the novelty of the test diminished.
5. Bright light is also one of the factors that increase the avoidance of the open arms.
6. The addition of ledges around the open arms reduces the component of anxiety to which the apparatus is sensitive.
7. The most important determinant is the method that is adopted for scoring animal's behaviour on the maze. Normally, percentages of the number and percentage of time for the open arms as a whole are considered.
8. The definition of what constitutes an arm entry is all important. Many determine that an arm entry has occurred only when the animal has exited the central square into one of the arms with all four paws. However, automated techniques that usually count light beam breaks to track the animals' progress around the maze are not sensitive to the position of the animals' paws.

9. The center square does not measure the same component of anxiety and need not to be scored as part of either the open or the closed arms.

1.3. OUTLINE OF PRESENT STUDY

The focus of the present study was to investigate the behaviour and plasma corticosterone changes of male rats subjected to physical and psychological stress. These animals were tested for behaviours (suggestive of anxiety and depression). The integrity of the neuro-endocrine system (plasma corticosterone response to acute challenges) and the effects of stress-restress to show possible sensitisation of the stress response, were also evaluated. Physical stress was achieved by subjecting the rats to repetitive foot shocks, while psychological stress was achieved by allowing other rats to witness the administration of physical stress in the adjacent chamber of a two-compartment communication box .

We hypothesized that the physical and psychological stress affect rats differently and these differences manifest in their respective behavioural and neuro-endocrine responses. The study therefore attempted to answer the following questions:

1. Do physical and psychological stress alter behaviour, and if so, are these alterations different?
2. Do physical and psychological stress lead to different or abnormal hypothalamic-pituitary-adrenal axis responses?
3. Do physical and psychological stress interact with the central serotonergic system?

CHAPTER 2

MATERIALS AND METHODS

2.1. INTRODUCTION

The communication box method is a useful model to investigate the physiological and endocrinological changes that occur under psychological stress, since it can produce an experimental anxiety based on intraspecies psychological communication without the direct physical stress (Oishi *et al.,* 2003). Takahashi *et al.* (1987) was the first to describe a box with only two compartments and in this procedure, non-foot shock rats are exposed to the visual, olfactory and auditory stimuli (such as vocalising, defecating, urinating and jumping) from foot shocked rats. Experiencing these psychological anxieties of foot shocked rats can induce an experimental anxiety in non-foot shocked rats. From many studies on the relationships between various stressors and its effect, it has become apparent that different stressors can have different effects on the rat depending on the nature, intensity and the time delay between stressors and the behaviour parameter studied.

Stress, defined as the response of the body to any threatening demand, can be divided into physiological responses, such as the activation of the neuro-endocrine system of the individual, and behavioural responses, that may include anxiety and fearful behaviour (Weninger *et al*., 1999). However, stress can also be divided into at least two broad categories namely physical stress or psychological stress. The

present study was therefore designed to investigate whether any differences exist in the body's response to either a physical stressor or a psychological stressor.

In order to do this investigation the following strategies were adopted:

1. a rat was subjected to a physical stressor
2. a separate animal was subjected to a psychological stressor
3. the behavioural responses in the respective animals were assessed
4. the neuro-endocrinological responses in the different animals were determined.

2.2. MATERIALS AND METHODS

2.2.1. ANIMALS AND HOUSING

Male Sprague Dawley rats were bred and housed in the animal facility of the University of Stellenbosch Medical School. Animals weighing 180 – 200 grams, at the beginning of the experiments were kept in pairs and were given standard laboratory rat chow and water *ad libitum*.

The housing facility humidity was kept at 80% and the day-night cycle was set at 06h00 lights on and 18h00 lights off. They were transported to the experimental room at least one-hour prior to the start of an experimental session. The animals were exposed to one of three conditions: physical stress, psychological stress or control conditions. During the entire duration of the experiment the rats were weighed every Monday, Wednesday and Friday to monitor the well-being of the

animals. The cages were cleaned twice a week. During the time period between the stress sessions and the behavioural tests the rats were housed in pairs and left undisturbed. Experimental procedures were approved by the Ethics Committee on Animal Experiments of the University of Stellenbosch.

2.2.2. STRESS PROCEDURES

The animal model used in this thesis to examine physical and psychological stress is based on the work of Pijlman in 2001. Two cage mate rats were placed in the two-compartment box (figure 2.1) for ten-minute sessions on one or five consecutive days. A perforated Perspex plate separated the two compartments to facilitate visual, auditory and olfactory communication. The rats were exposed to a physical-, psychological stress or a non-stress condition.

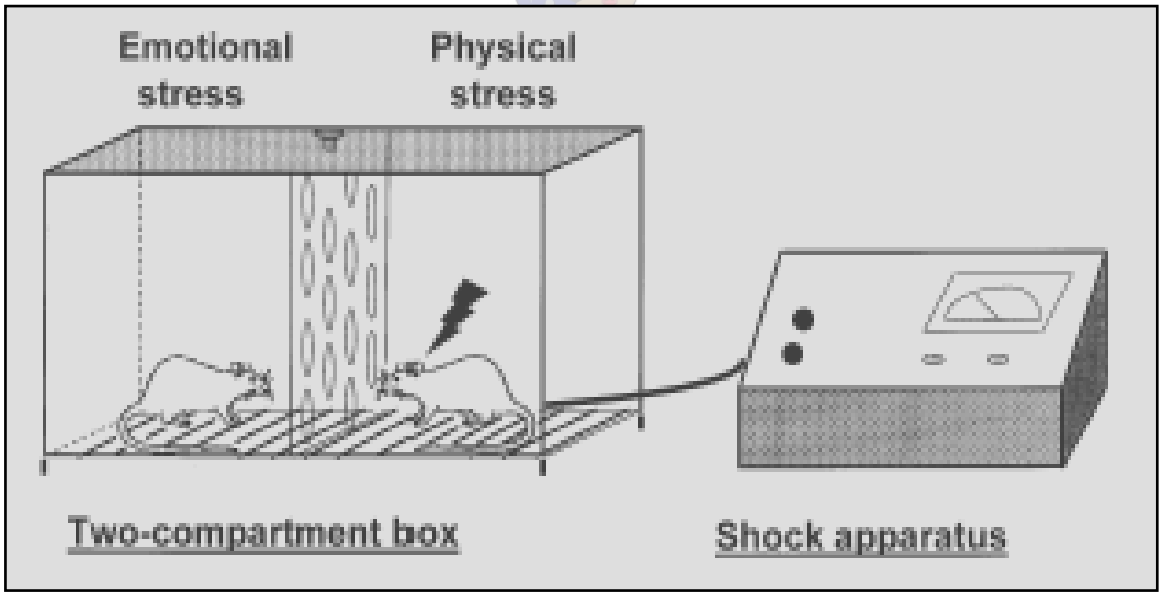


Figure 2.1: Two-compartment box (Pijlman, 2001).

Physical stress consisted of ten unpredicted foot shocks (0.5mA for 1 second), delivered at random in one compartment. Random electrical currents were conducted through the metal grid floor of the compartment, generated by a shock generator and scrambler. An example of the time periods was at 0:50, 1:20, 2:30, 3:45, 4:40, 5:35, 6:55, 7:45, 8:30 and 9:50 minutes.

To induce psychological stress, animals were placed in a compartment adjacent to the electric foot shocked animals in the same communication box. These animals were not given any electric foot shocks. They were present in the adjacent compartment during the foot shock treatment and were exposed to visual, auditory and olfactory sensations from the electric foot shocked rats.

Control animals were placed in pairs in the separate compartments, without receiving any shocks. The two-compartment box was cleaned with 70% ethanol after each pair of rats had been removed.

Open field and elevated plus-maze activities were consecutively recorded, for five minutes on video, 5 or 10 days after the last stress session. Evaluators, blind to the status of the animals, rated the video recordings afterwards. Each animal was placed in the apparatus in the same position and the following behaviours were assessed in the open field: rearing (standing on hind legs), grooming and time spend in the inner zone and time spends in the outer zone of the open field. All four paws had to be over the demarcated lines to constitute a zone crossing. In the

elevated plus-maze, rearing (standing on hind legs), grooming and time spend in the open, closed arms and center, as well as the number of entries in open arms and closed arms, were documented.

In order to evaluate the integrity of the hypothalamic-pituitary-adrenal axis of stressed animals, rats were individually placed in a restrainer for 10 minutes and blood was collected at 15 and 60 minutes post-stress for endocrinological determinations.

2.2.3. ENDOCRINE RESPONSE

The plasma corticosterone concentration was measured to provide an endocrinological index of the animal's stress response. One day (24 hours) after the open field activity and elevated plus-maze tests were performed, the rats were decapitated and blood was collected in chilled centrifuge tubes containing 200 micro liter of 125 millimolar EDTA as anticoagulant. After centrifugation (2500 revolutions per minute for 10 minutes at 4º C), supernatants were removed and aliquoted for storage in liquid nitrogen.

Corticosterone concentrations were determined using a commercially available corticosterone radioimmuno-assay kit (ImmuChem, Hamburg, Germany). The principle of the assay is based on the measurement of the concentration of antigen molecules as determined by the extent to which it combines with its antibody in the presence of a radioactively labeled analog.

In the assay, a limited amount of specific antibody is reacted with the corresponding hormone labeled with a radioisotope. Upon addition of an increasing amount of the corticosterone, a corresponding decreasing fraction of the radioisotope hormone is bound to the antibody. After separation of the bound for the free corticosterone, the amount of radioactivity in one or both of these fractions was determined (Table 2.1) and used to construct a standard curve against which the unknown samples were measured. Corticosterone determinations were done using a Gamma counter (Cobra: Frankfurt, Germany). Figure 2.2 is an example of a standard curve (adapted from one of the experiments done).

SAMPLE	CPM	AVG. CPM	AVG.-NSB CPM	%B/Bo	RESULT (ng/mL)
NSB (blank)	1085 917	1001			
0 ng/mL	21411 21083	21247	20246	100	
25 ng/mL	19165 18601	18883	17882	88	
50 ng/mL	17142 17026	17084	16083	79	
100 ng/mL	14392 13971	14181	13180	65	
250 ng/mL	10335 10542	10438	9437	47	
500 ng/mL	7363 7366	7364	6363	31	
1000 ng/mL	4880 5046	4963	3962	20	
Control I	9286 9261	9273	8272	41	320
Control II	12356 12228	12292	11291	56	160
Control III	17598 18007	17802	16801	83	38

Table 2.1: Standard results

2.2.3.1. CALCULATIONS

The averages of all duplicated tubes were taken and the averaged NSB (blank) counts were subtracted from the averages obtained. This yielded the corrected

values. The corrected values were divided by the corrected zero calibrator value to obtain the percent bound. The formula used was:

$$\%B/Bo = \frac{\text{CPM (sample)} - \text{CPM (NSB)}}{\text{CPM (0 calibrator)} - \text{CPM (NSB)}} \times 100$$

CPM = Average counts of duplicates

Sample = Particular serum or calibrator being calculated

NSB = Non-specific binding tube (also known as blank tube)

0 Calibrator = 0 tube (also known as the 100% binding tube or total binding tube).

The percentage bound is plotted against the concentration of corticosterone for all the standards (25 – 1000 nanogram/milliliter) and this yielded the calibrator curve. The sample values are then read directly from this curve.

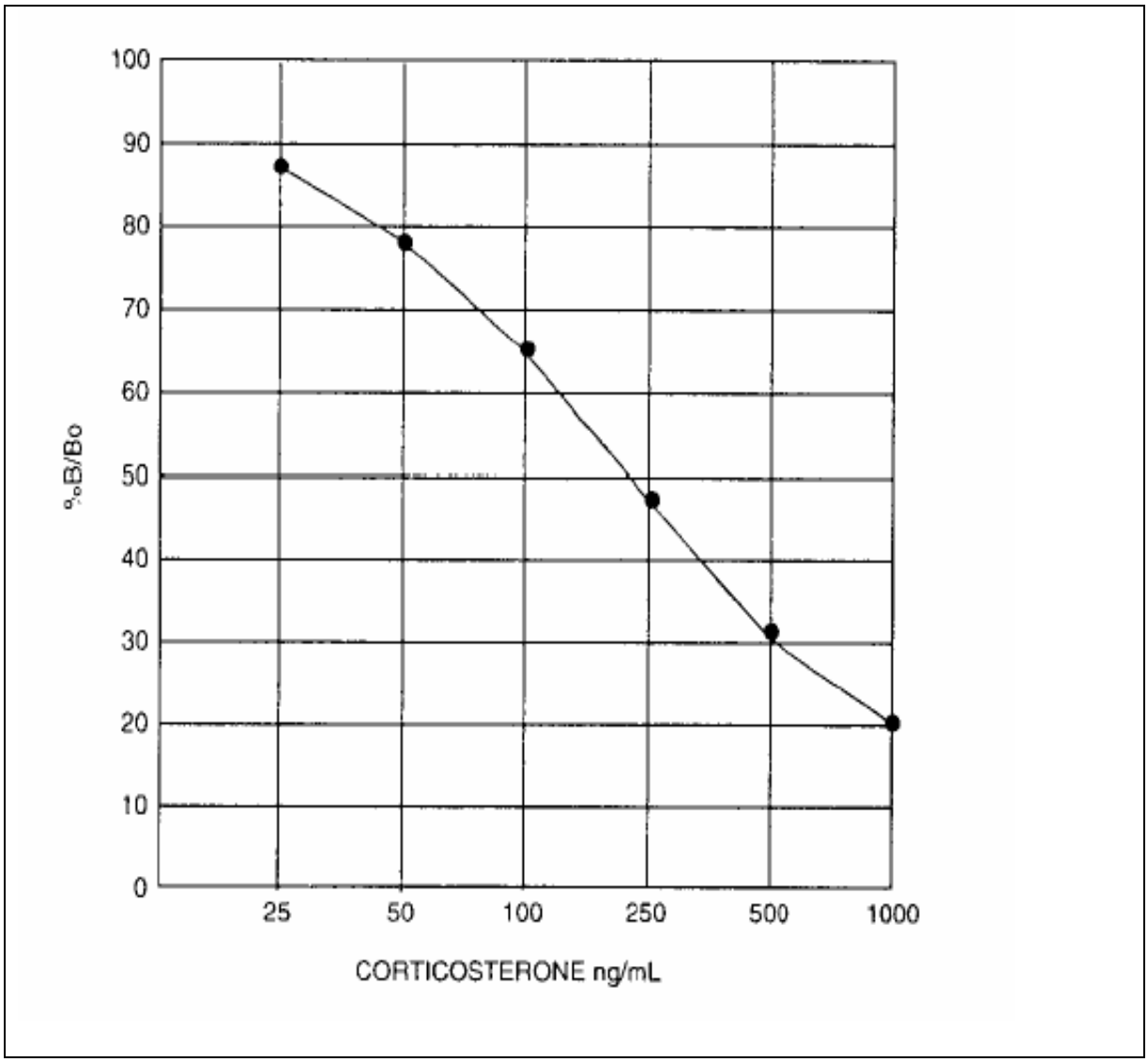


Figure 2.2: Example of a standard curve

2.2.4. BEHAVIOURAL OBSERVATIONS

2.2.4.1. THE OPEN FIELD

The behavioural responses were assessed during a 5-minute video recording using an open field apparatus. The open field is built from opaque plexiglass with white walls (50 centimeter) and a light gray floor (100 x 100 centimeter). The quadrants

and zones on the floor were marked with white and yellow tape and the outer zone was specified at 15 centimeter from the walls. The remaining area in the center served as the inner zone. Quadrants of 20 x 20 centimeters were demarcated throughout the arena (figure 2.3). The following parameters were noted to indicate the level of anxious behaviour in the animals – rearing, grooming, time spent in inner or outer zone and number of blocks crossed (Pietersen, 2001).

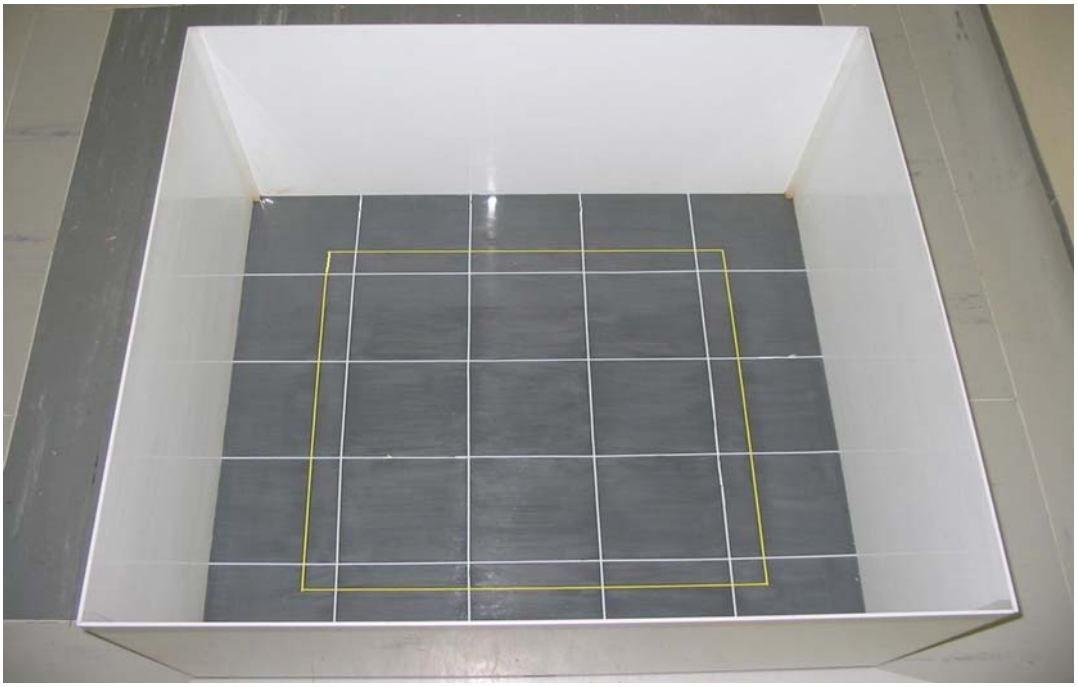


Figure 2.3: The open field.

2.2.4.2. THE ELEVATED-PLUS MAZE

The elevated-plus maze was initially validated by Pellow *et al*., (1985a) and has subsequently been validated in our laboratory by Richter, (2003) and Pietersen, (2001). It is widely used in rat behavioural studies as a measure of locomotor

activity and anxiety. This provides a novel environment that a rat would like to explore, but the rat simultaneously experiences fear of the open spaces and height.

Figure 2.4: The elevated plus-maze

The elevated plus-maze is constructed of black Perspex (figure 2.4). It consists of two open arms and two arms that are enclosed by high walls. The open arms are perpendicular to the closed arms, with four arms intersecting to form the shape of a plus sign. The elevated plus-maze is usually elevated approximately 50 cm above the floor. Security is provided by the closed arms while the open arms offer exploratory value. Therefore, one might expect anxious rats to spend less time in the open arms than those that are less fearful. When placed in an elevated plus-maze for the first time, a rat's behaviour is largely based on its anxiety level.

Normal rats that have not received any anti-anxiety drugs will become moderately anxious in this new environment. Less anxious rats will spend more time in open arms compared to normal rats (Treit *et al.,* 1993).

2.2.5. EXPERIMENTAL PROTOCOLS

Experiment 1: (1-day stress, 5-day behaviour)

This group of rats was stressed for 1 day only. 5 days after the stress session open field and elevated plus-maze activity was assessed for five minutes each consecutively. The following day the rats were decapitated and blood was collected.

Experiment 2: (5-day stress, 5-day behaviour)

Rats were stressed for 5 consecutive days. Open field activity and elevated plus-maze activity was assessed 5 days after the last stress session. The following day the rats were decapitated and blood was collected.

(5-day stress, 5 days, restrain 10 min, decapitation after 15 min/60 min)

Rats were stressed for 5 days, after which a further 5 days were waited before the animals were restrained for 10 minutes in a restrainer. The one group of animals was subsequently decapitated for blood collection at 15 minutes and the other group at 60 minutes post-stress.

Experiment 3: (5-day stress, 10-day behaviour)

This group of rats was treated similarly to the previous group i.e. stressed for 5 days. However, in this group the open field activity and elevated plus-maze activity were assessed 10 days after the last stress session. The following day the rats were decapitated and blood was collected.

Experiment 4: (5-day stress, 10-day behaviour, 10-day Citalopram treatment)

After 5 days of stress, the physical and psychological stressed rats received a dose of Citalopram (10milligram/kilogram) daily intraperitoneal for the 10 days. Open field activity and elevated plus-maze activity were assessed for five minutes consecutively and the following day the rats were decapitated and blood was collected.

Experiment 5 : (5-day stress, restress and 10-day behaviour)

For this experiment the animals were stressed for 5 days and subjected to the same stress for one episode, 5 days after the last stress. Open field activity and elevated plus-maze activity were assessed, 5 days after this re-stress period. The following day the rats were decapitated and blood was collected.

Experiment 6: (1-day physical and psychological stress, 4-day physical stress, 5-day behaviour)

On the first day of being stressed, both the physical and psychological stressed rats were subjected to electric foot shock. The next 4 days only one group was

exposed to the physically stressed rats, while the other group was subjected to psychological stress. Open field activity and elevated plus-maze activity were again assessed 5 days after the last stress session. The following day the rats were decapitated and blood was collected.

2.2.6. STATISTICS

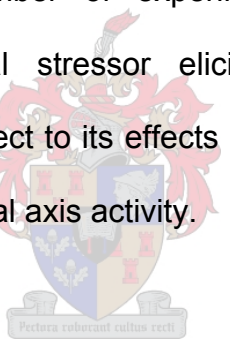
All data are presented as means with standard error of the mean. Significant differences between the groups (physical, psychological and control) were tested using factorial analysis of variance by a statistician from this university. A non-parametric, Levene test of homogeneity of variances was done and the Kruskal Wallis test was performed. Finally, the Dunn's multiple comparison posttest was used to assess significance of differences between groups, e.g. physical versus psychological or psychological versus control. Differences between the experimental groups were considered to be significant if $p \leq 0.05$.

CHAPTER 3

RESULTS

3.1. INTRODUCTION

The response of the body to a single stressor is complex, involving a carefully orchestrated reaction between central neurotransmitter and neuro-endocrine systems. This complexity is further expanded by the fact that individuals are constantly subjected to various types of stress. The question therefore arose as to whether differences existed in the body's neurobiological response to different stressors. Subsequently a number of experiments have been designed to investigate whether a physical stressor elicits a different response to a psychological stressor, with respect to its effects on specific behavioural outcomes and hypothalamic-pituitary-adrenal axis activity.



3.2. EXPERIMENT 1: (1-day stress, 5-day behaviour)

A group of 26 rats was subjected to 1 day of stress and 5 days after this stress session, open field activity and elevated plus-maze activity were used to assess the behaviour of the animals in this experiment. The results in Table 3.1 show that the rats exposed to physical stress showed an increase in grooming when in the open field. This increase was significant in comparison to the psychologically stressed animals and is graphically depicted in Figure 3.1. There was no difference between the amounts of grooming in the psychological group compared to the controls. No other significant differences were observed in any of the parameters of the

elevated-plus maze (Table 3.1). Neuro-endocrinological assessment of the hypothalamic-pituitary-adrenal axis showed that there was no significant difference between the corticosterone levels of the three groups of rats studied (Figure 3.2).

TABLE 3.1: 1-DAY-STRESS, 5-DAY BEHAVIOUR

		Physical	Psychological	Control	p-value
	n = 26	9	9	8	
OPEN FIELD					
REARING	MEANS	3.67	4.22	2.63	p ≥ 0.05
	SEM	±1.07	±1.27	±0.99	
GROOMING	MEANS	**3.11**	**1.00**	1.25	**p ≤ 0.01**
	SEM	**±0.59**	**±0.41**	±0.31	
TIME IN INNER ZONE (sec)	MEANS	0.89	8.11	0	p ≥ 0.05
	SEM	±0.61	±8.11	0	
TIME IN OUTER ZONE (sec)	MEANS	299.11	291.89	300	p ≥ 0.05
	SEM	±0.61	±8.11	0	
NUMBER OF BLOCKS	MEANS	28.67	48.22	37.88	p ≥ 0.05
	SEM	±8.17	±14.06	±9.11	
ELEVATED PLUS					
REARING	MEANS	3.11	2.11	1.38	p ≥ 0.05
	SEM	±1.16	±0.82	±0.57	
GROOMING	MEANS	2.22	2.11	1.38	p ≥ 0.05
	SEM	±0.70	±0.54	±0.32	
TIME IN OPEN ARMS (sec)	MEANS	8.89	10.33	9.63	p ≥ 0.05
	SEM	±6.32	±4.85	±5.61	
TIME IN CLOSED ARMS (sec)	MEANS	282.56	276.44	286.5	p ≥ 0.05
	SEM	±6.66	±9.26	±5.82	
ENTRIES IN OPEN ARMS	MEANS	1.22	1.67	0.88	p ≥ 0.05
	SEM	±0.66	±0.96	±0.44	
ENTRIES IN CLOSED ARMS	MEANS	7.44	7.67	8.25	p ≥ 0.05
	SEM	±1.03	±2.30	±1.67	

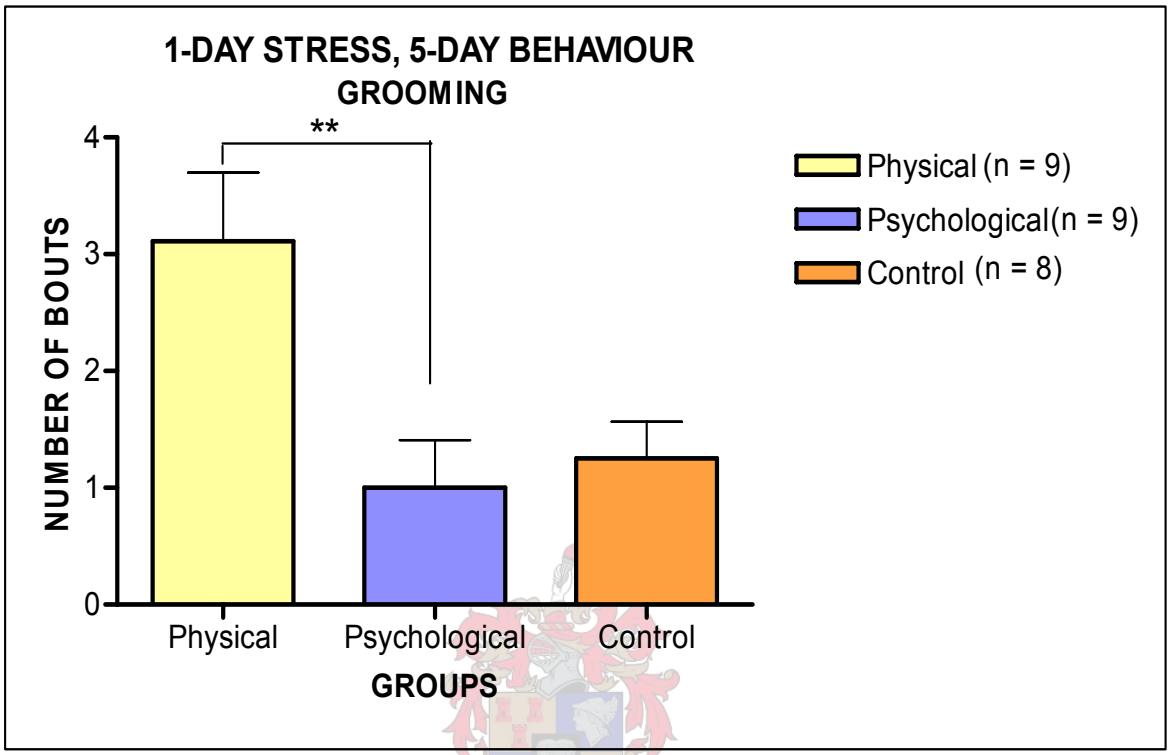


Figure 3.1. Grooming behaviour during the open field test. The animals were subjected to 1 day stress and their behaviour was assessed 5 days later.

Values are shown as means ±SEM. ** $p \leq 0.01$; physically stressed group was significantly different from psychologically stressed group.

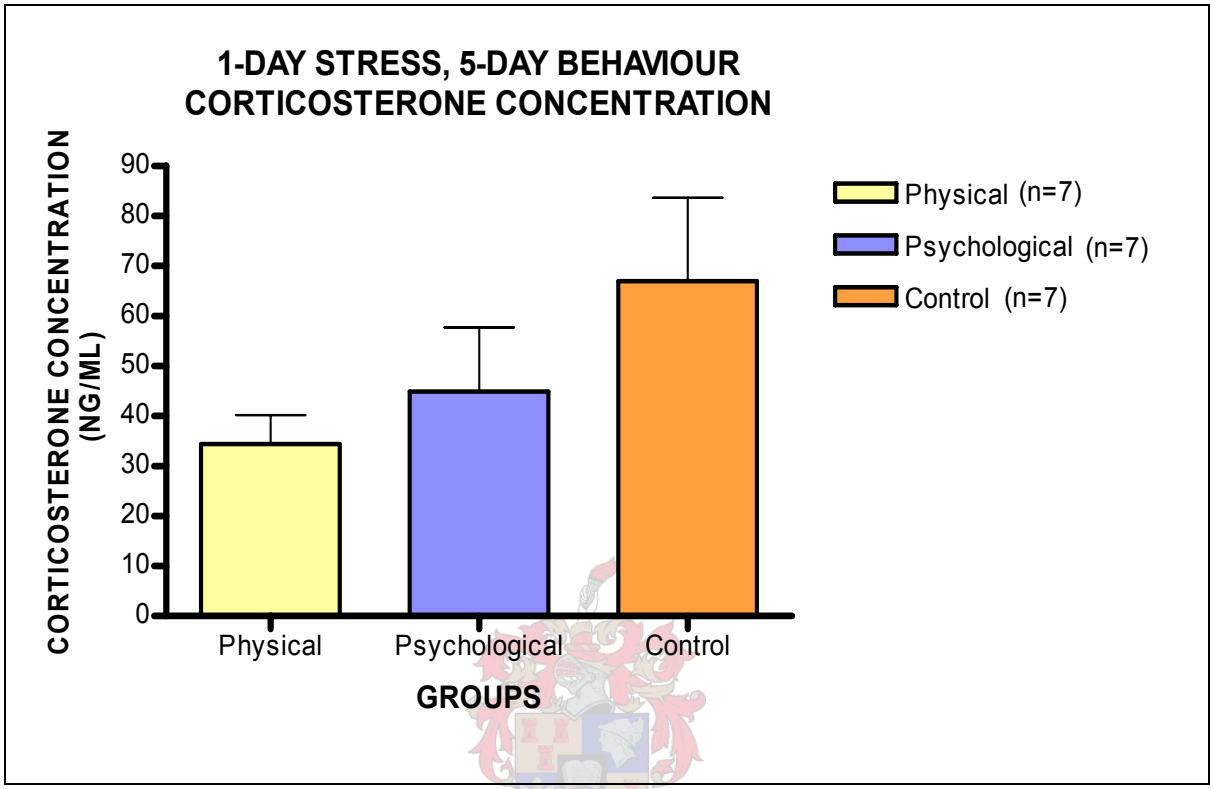


Figure 3.2. Basal plasma corticosterone concentrations. The animals were subjected to 1 day stress and trunk blood was collected 6 days later. Values are shown as means ±SEM. None of these groups is significantly different from each other.

3.3. EXPERIMENT 2: (5-day stress, 5-day behaviour)

Following our initial results in Experiment 1, the stress protocol was adapted so that rats in this experiment were subjected to 5 days of stress. Table 3.2 shows that increasing the stress period from 1 day to 5 days, resulted in a significant reduction in rearing behaviour in rats that were physically stressed (Figure 3.3). There was no significant difference in the levels of rearing between the control and psychologically stressed group of animals. Similar to Experiment 1 no significant differences were observed in the parameters of the elevated-plus maze (Table 3.2).

Trunk blood was collected from these animals for basal corticosterone concentration determinations. From Figure 3.4 it is obvious that no significant difference in plasma corticosterone levels existed for any of the groups tested at baseline levels. However, the increase in corticosterone concentration 15 minutes after being subjected to subsequent restraint stress, was significantly reduced in both the physically stressed and the psychologically stressed groups when compared to controls. At 60 minutes, the plasma corticosterone concentrations of all three groups returned to comparable levels.

TABLE 3.2: 5-DAY-STRESS, 5-DAY BEHAVIOUR

		Physical	Psychological	Control	p-value
	n = 30	10	10	10	
OPEN FIELD					
REARING	MEANS	**5.220**	**12.443**	**11.059**	**p ≤ 0.05**
	SEM	**±1.937**	**±2.817**	**±2.152**	
GROOMING	MEANS	1.200	1.200	1.400	p ≥ 0.05
	SEM	±0.359	±0.327	±0.427	
TIME IN INNER ZONE (sec)	MEANS	7.700	8.800	11.500	p ≥ 0.05
	SEM	±4.045	±3.369	±3.229	
TIME IN OUTER ZONE (sec)	MEANS	292.3	291.2	288.5	p ≥ 0.05
	SEM	±4.045	±3.369	±3.229	
NUMBER OF BLOCKS	MEANS	87.900	89.200	99.500	p ≥ 0.05
	SEM	±13.457	±15.038	±12.364	
ELEVATED PLUS					
REARING	MEANS	6.000	5.100	5.800	p ≥ 0.05
	SEM	±1.145	±0.849	±1.019	
GROOMING	MEANS	2.800	1.700	1.100	p ≥ 0.05
	SEM	±0.772	±0.496	±0.315	
TIME IN OPEN ARMS (sec)	MEANS	33.000	33.200	33.100	p ≥ 0.05
	SEM	±8.517	±6.766	±8.007	
TIME IN CLOSED ARMS (sec)	MEANS	249.2	246.4	249	p ≥ 0.05
	SEM	±10.001	±8.423	±8.764	
ENTRIES IN OPEN ARMS	MEANS	3.900	3.400	3.500	p ≥ 0.05
	SEM	±0.924	±0.733	±0.946	
ENTRIES IN CLOSED ARMS	MEANS	9.700	10.100	7.600	p ≥ 0.05
	SEM	±1.012	±0.795	±0.601	

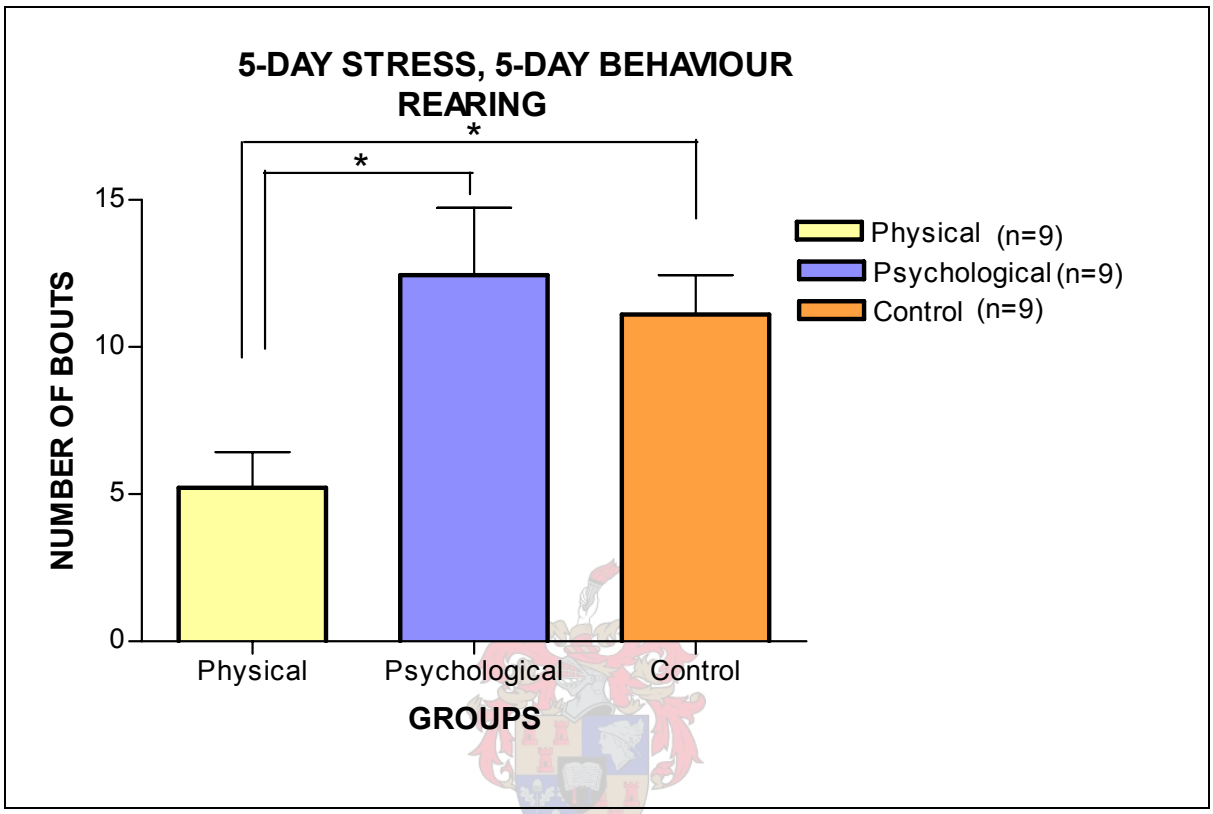


Figure 3.3. Rearing behaviour during the open field test. The animals were subjected to 5 days stress and their behaviour evaluated 5 days later. Values as shown as means ±SEM. * p< 0.05; physically stressed group significantly different from psychologically stressed and control group respectively.

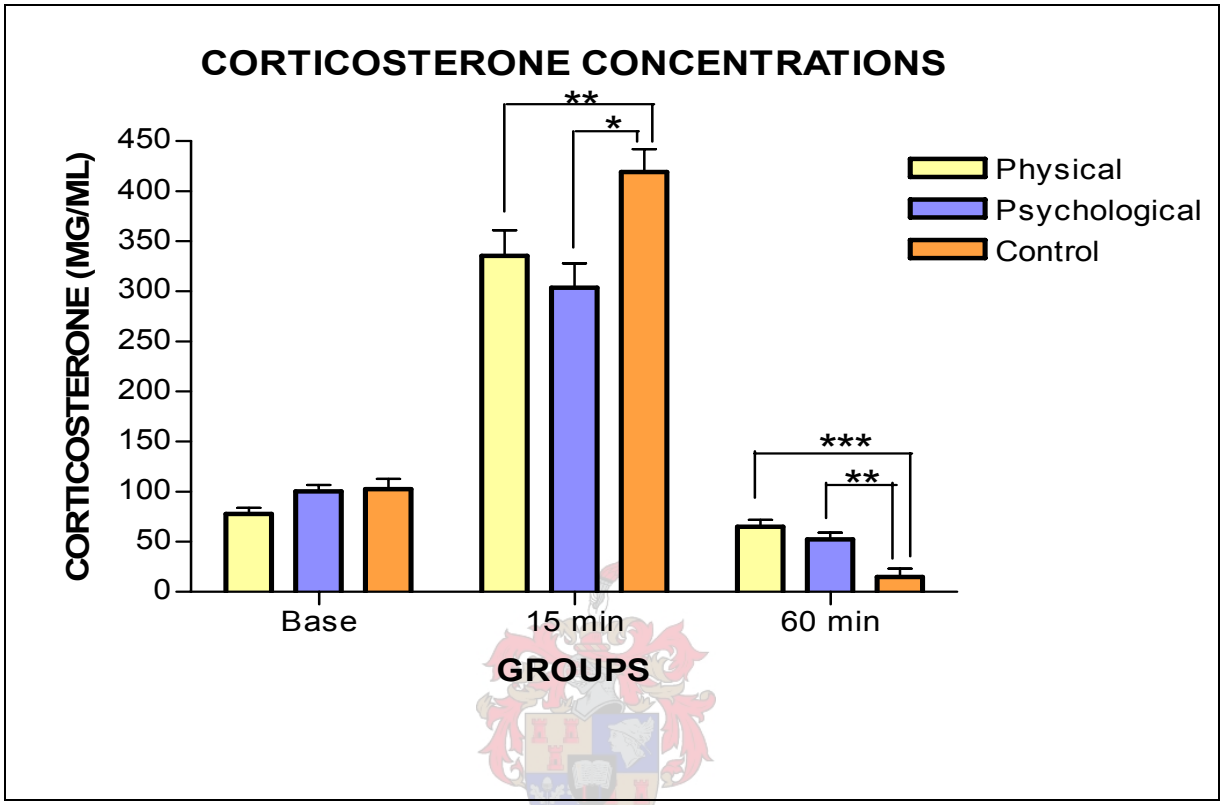
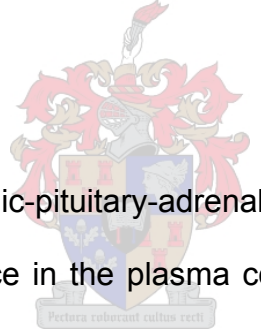


Figure 3.4. Corticosterone concentrations of the 5-day stress, 5-day behaviour experiment. Corticosterone concentrations were measured at baseline (n=30), as well as 15 minutes (n = 22) and 60 (n = 20) minutes after restraint stress. Values are shown as means ±SEM. ** p< 0.01; *** p< 0.001; There is a significant difference between the physical stress and control rats and between the psychological stress and control rats in the 15 minute and the 60 minute experiments.

3.4. EXPERIMENT 3: (5-day stress, 10-day behaviour)

In our quest to optimize our assessment time interval, we evaluated the behaviour of another group of animals (that were stressed for 5 days) 10 days after the exposure to the last stress (Table 3.4). This protocol yielded a number of remarkable findings in that significant differences between the physically stressed and the control rats in rearing (Figure 3.10), percentage time in the inner (Figure 3.11) and outer zone (Figure 3.12), as well as the number of blocks crossed (Figure 3.13), were observed in the open field test. In addition, on the elevated-plus maze a significant difference between the psychologically stressed rats and control rats was obtained in the percentage of entries in open arms is shown in Figure 3.14.



Evaluation of the hypothalamic-pituitary-adrenal axis activity of these animals showed no significant difference in the plasma corticosterone levels of any of the groups tested (Figure 3.15).

TABLE 3.3: 5-DAY-STRESS, 10-DAY BEHAVIOUR

		Physical	Psychological	Control	p-value
	n = 24	9	9	6	
OPEN FIELD					
REARING	MEANS	**2.667**	5.556	**9.667**	**p ≤ 0.05**
	SEM	**±0.972**	±1.980	**±2.061**	
GROOMING	MEANS	1.333	1.222	2.000	p ≥ 0.05
	SEM	±0.408	±0.222	±0.683	
TIME IN INNER ZONE (sec)	MEANS	**0.111**	2.111	**4.500**	**p ≤ 0.05**
	SEM	**±0.111**	±1.532	**±1.689**	
TIME IN OUTER ZONE (sec)	MEANS	**299.889**	297.889	**295.5**	**p ≤ 0.05**
	SEM	**±0.111**	±1.532	**±1.689**	
NUMBER OF BLOCKS	MEANS	**22.143**	**81.429**	**88.667**	**p ≤ 0.05**
	SEM	**±8.086**	**±16.435**	**±11.592**	
ELEVATED PLUS					
REARING	MEANS	3.000	4.333	4.167	p ≥ 0.05
	SEM	±0.687	±1.190	±1.046	
GROOMING	MEANS	1.222	1.778	1.333	p ≥ 0.05
	SEM	±0.278	±0.465	±0.495	
TIME IN OPEN ARMS (sec)	MEANS	37.667	41.889	58.33	p ≥ 0.05
	SEM	±8.796	±7.453	±6.261	
TIME IN CLOSED ARMS (sec)	MEANS	254.667	247.444	239.67	p ≥ 0.05
	SEM	±10.235	±10.332	±4.617	
ENTRIES IN OPEN ARMS	MEANS	4.556	**4.444**	**8.667**	**p ≤ 0.05**
	SEM	±1.132	**±0.784**	**±1.022**	
ENTRIES IN CLOSED ARMS	MEANS	10.333	8.111	9.333	p ≥ 0.05
	SEM	±1.000	±1.047	±0.882	

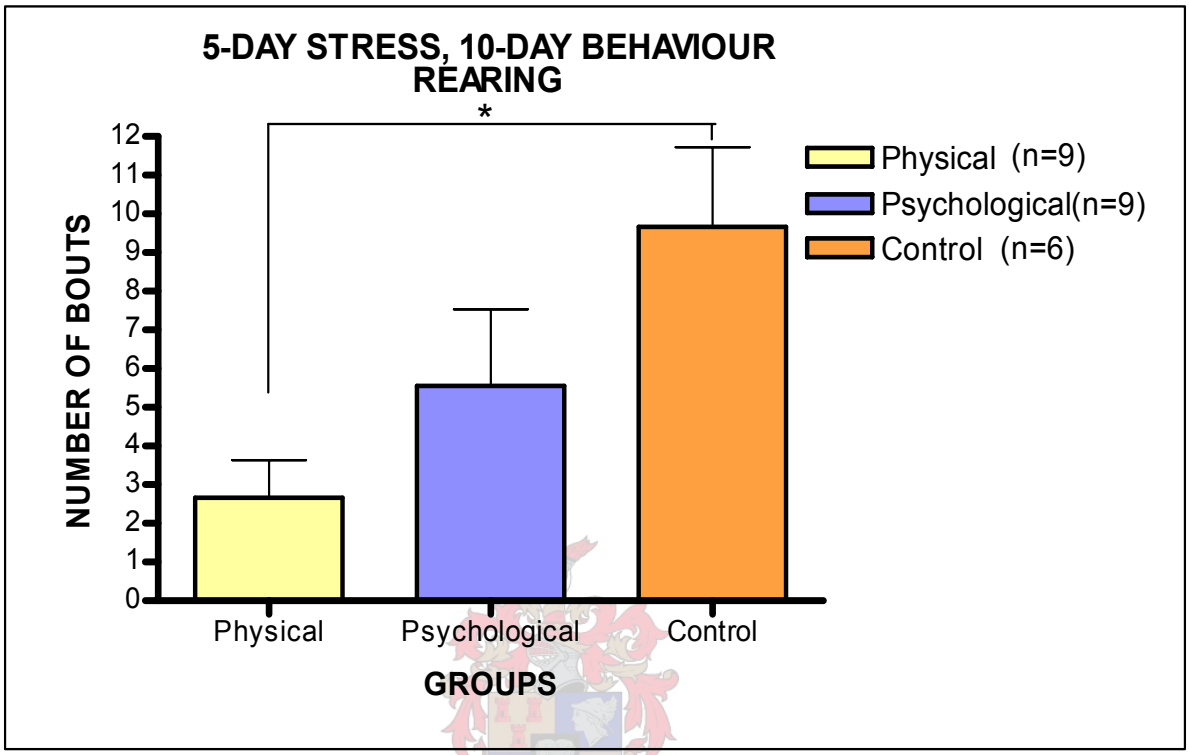


Figure 3.5. Amount of rearing in the open field. The animals were subjected to 5 day stress and behaviour was done 10 days after that. Values are shown as means ± SEM. * $p \leq 0.05$; the physically stressed group was significantly different from the control group.

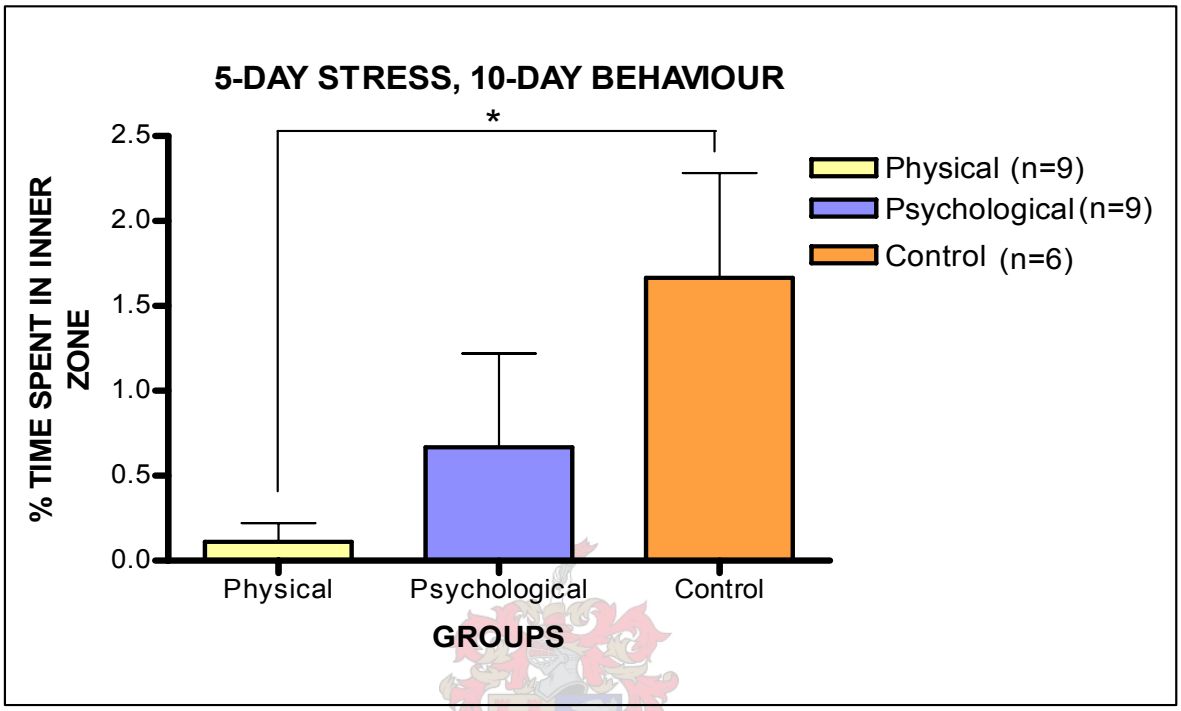


Figure 3.6. Percentage time spent in the inner zone in the open field. The animal groups were stressed for 5 days and behaviour was done 10 days after that. Values are shown as means ± SEM. * $p \leq 0.05$; the physically stressed group was significantly different from the control group.

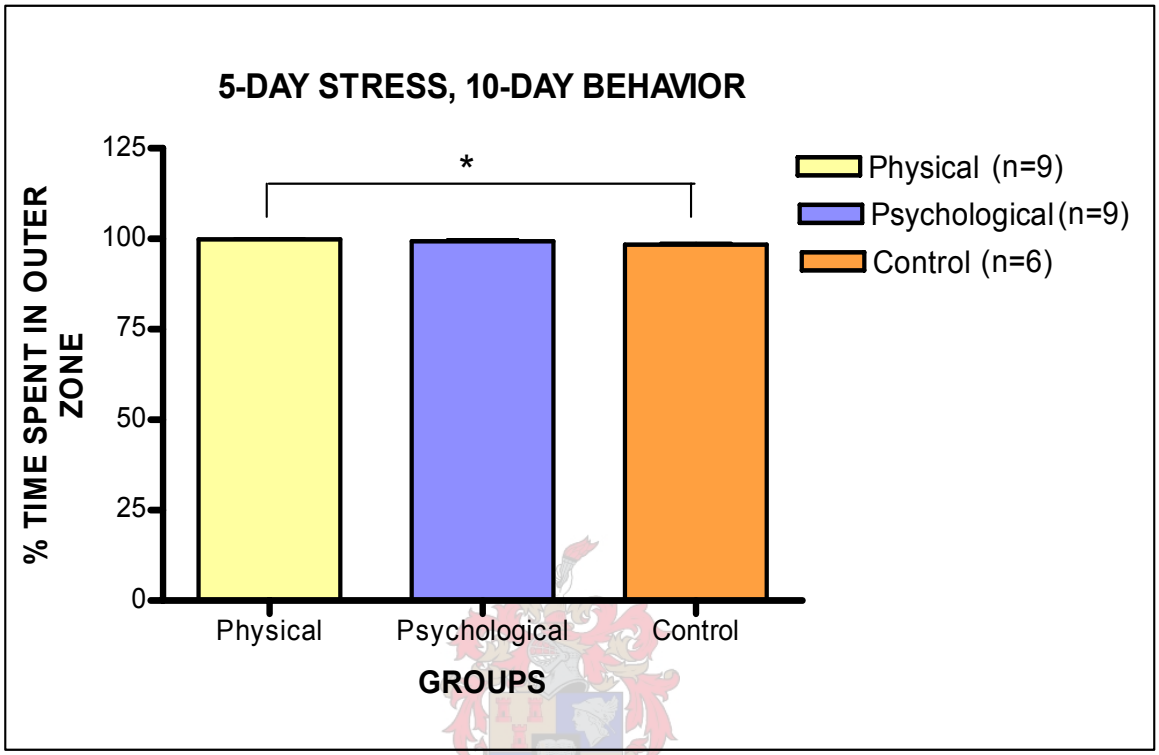


Figure 3.7. Percentage time spent in the outer zone in the open field. The animal groups were stressed for 5 days and behaviour was done 10 days after that. Values are shown as means ± SEM. * $p \leq 0.05$; physically stressed group was significantly different from the control group.

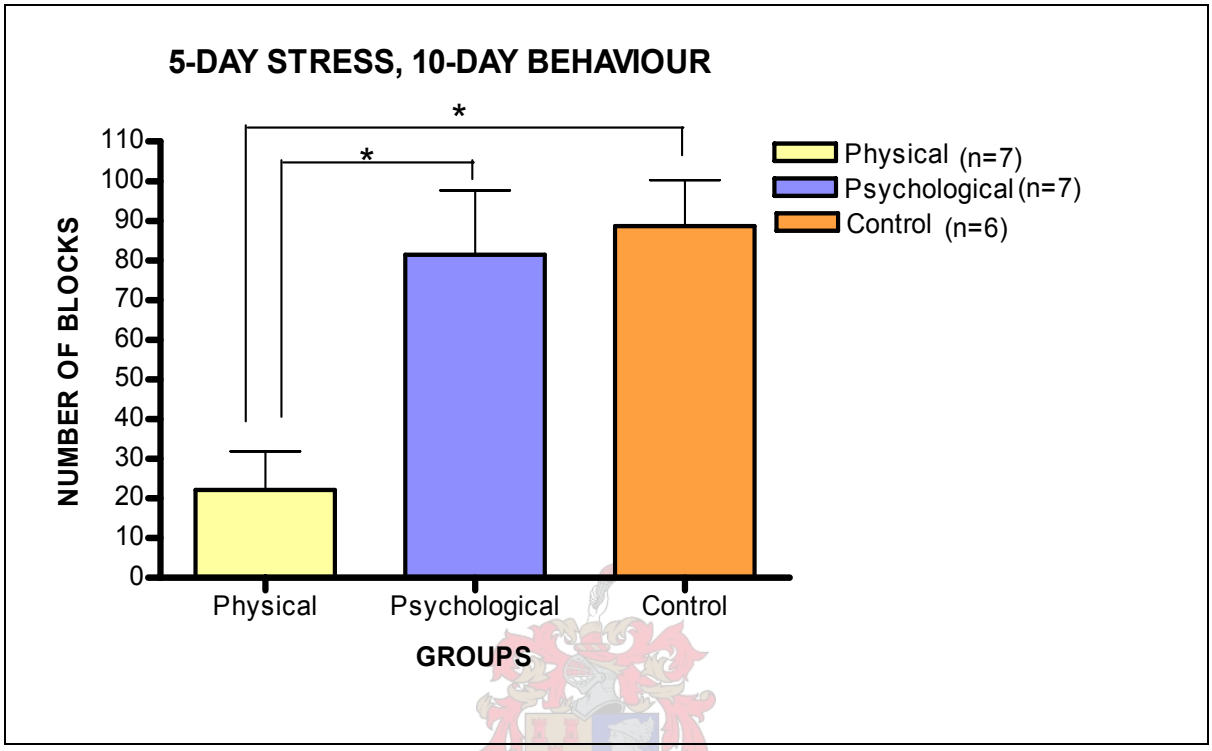


Figure 3.8. Number of blocks crossed in the open field. The animal groups were stressed for 5 days and behaviour was done 10 days after that. Values are shown as means ± SEM. * $p \leq 0.05$; There was a significant difference between the physical versus the psychological group, as well as between the physical and the control group.

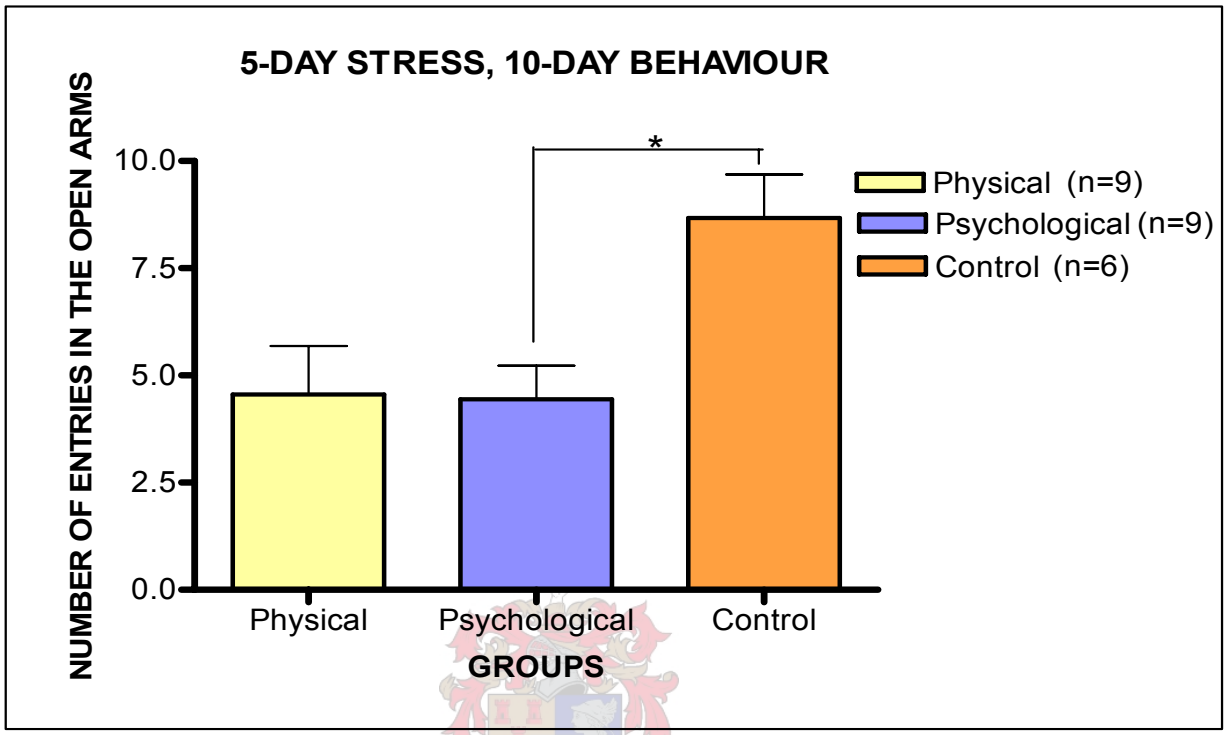


Figure 3.9. Number of entries in the open arms in the elevated plus-maze. The animal groups were stressed for 5 days and behaviour was done 10 days after that. Values are shown as means ± SEM. * $p \leq 0.05$; There was a significant difference between the psychological versus the control group.

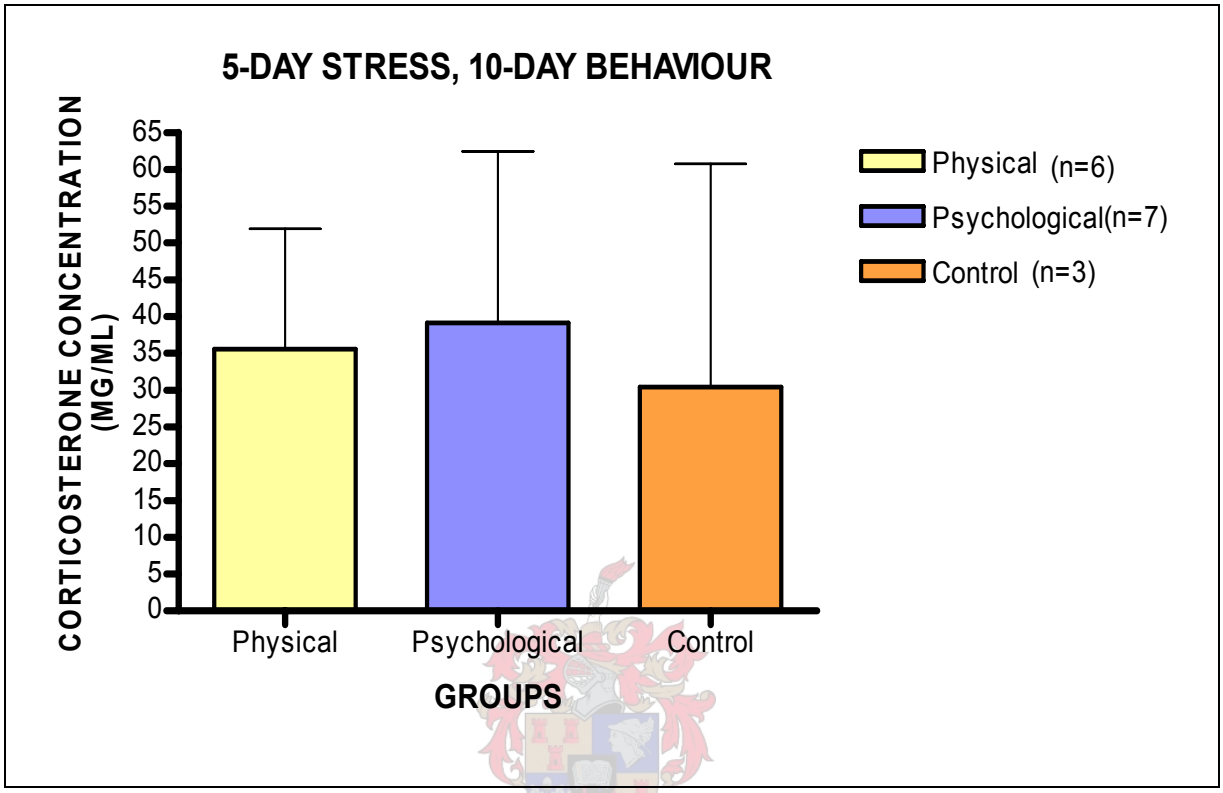


Figure 3.10. Corticosterone concentration of an experiment where the animal groups were stressed for 5 days and behaviour was done 10 days after that. Values are shown as means ± SEM. There was no significant difference between any of the groups.

3.5. EXPERIMENT 4: (5-day stress, 10-day behaviour, 10-day Citalopram treatment)

The selective serotonin reuptake inhibitor, Citalopram, is commonly used to treat a number of psychiatric disorders such as anxiety and mood disorders. The next experiment was performed to see whether a daily dose of Citalopram (10mg/kg, intraperitoneally) for 10 days, was able to reverse the behavioural abnormalities observed in previous experiments. Table 3.6 therefore shows the behavioral results of rats that were subjected to 5 days of stress and received their Citalopram regimen for the next 10 days. Rats exposed to psychological stress showed a significant increase in crossing the number of blocks, in comparison with the physical stressed rats, in Figure 3.18. Treating rats with Citalopram immediately following the stress period, abolished this difference.

However, there was no significant difference in the basal corticosterone concentrations of these rats, whether they received Citalopram or not (Figure 3.19).

TABLE 3.4: 5-DAY-STRESS, 10-DAY BEHAVIOUR AND 5-DAY-STRESS, 10-DAY BEHAVIOUR AND 10-DAY TREATMENT WITH CITALOPRAM.

		10-DAY CITALOPRAM			10-DAY BEHAVIOUR		
		Physical	Psychological		Physical	Psychological	p-value
		11	11		9	9	
OPEN FIELD							
REARING	MEANS	4.545	6.000		2.667	5.556	p ≥ 0.05
	SEM	±1.209	±1.809		±0.972	±1.980	
GROOMING	MEANS	2.091	2.636		1.333	1.222	p ≥ 0.05
	SEM	±0.392	±0.453		±0.408	±0.222	
TIME IN INNER ZONE (sec)	MEANS	1.455	3.909		0.111	2.111	p ≥ 0.05
	SEM	±0.755	±2.852		±0.111	±1.532	
TIME IN OUTER ZONE (sec)	MEANS	298.545	296.091		299.889	297.889	p ≥ 0.05
	SEM	±0.755	±2.852		±0.111	±1.532	
NUMBER OF BLOCKS	MEANS	35.909	51.545		**22.143**	**81.429**	**p ≤ 0.05**
	SEM	±12.288	±12.856		**±8.086**	**±16.435**	
ELEVATED PLUS							
REARING	MEANS	3.273	2.727		3	4.333	p ≥ 0.05
	SEM	±0.787	±0.702		±0.687	±1.190	
GROOMING	MEANS	1.636	2.364		1.222	1.778	p ≥ 0.05
	SEM	±0.364	±0.432		±0.278	±0.465	
TIME IN OPEN ARMS (sec)	MEANS	13.273	21.182		37.667	41.889	p ≥ 0.05
	SEM	±4.586	±6.143		±8.796	±7.453	
TIME IN CLOSED ARMS (sec)	MEANS	273.182	257.909		254.667	247.444	p ≥ 0.05
	SEM	±4.975	±9.032		±10.235	±10.332	
ENTRIES IN OPEN ARMS	MEANS	1.000	2.000		4.556	4.444	p ≥ 0.05
	SEM	±0.330	±0.556		±1.132	±0.784	
ENTRIES IN CLOSED ARMS	MEANS	6.091	6.455		10.333	8.111	p ≥ 0.05
	SEM	±1.148	±0.813		±1.000	±1.047	

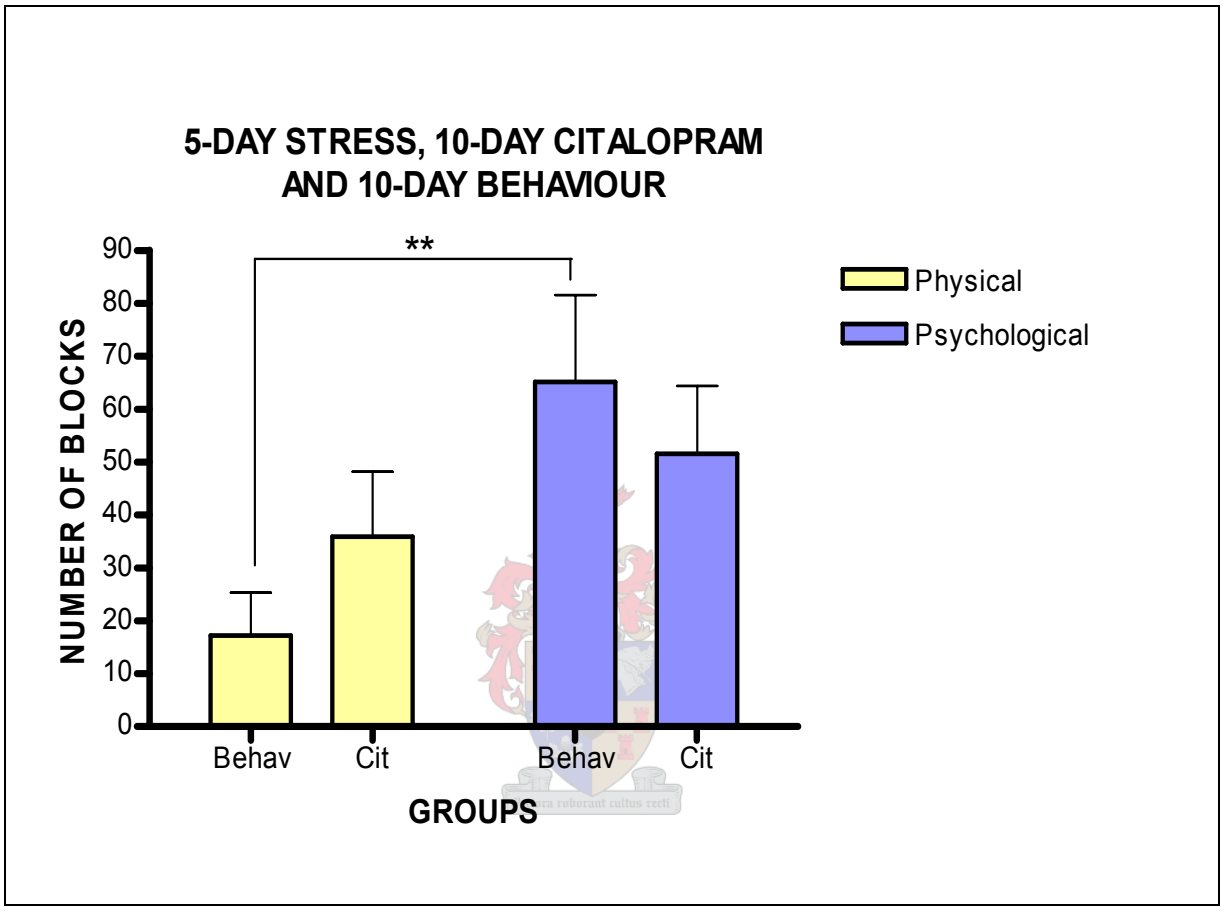


Figure 3.11. Number of blocks crossed in the open field. Two groups of rats were stressed for 5 days and behaviour was done 10 days after that. One of the two groups was treated with Citalopram for 10 days. Values are shown as means ±SEM. ** $p \leq 0.01$; There was a significant difference between the physical and psychological stressed rats in the experiment where the rats were not treated with Citalopram.

3.6. EXPERIMENT 5: (5-day stress, restress and 10-day behaviour)

Whilst the results of Experiment 4 suggested that 10 days after the last stress session was a better time point to study behavioural changes, we wondered whether an additional stress session would elicit any supplementary effects. Subsequently a separate group of rats that were stressed for 5 days, received another stress session 5 days after the last stressed session, but also 5 days prior to being tested. It was argued that this additional stress session would serve as a reminder to the animal of the initial stress sessions. Open field activity and elevated-plus maze activity were consequently assessed 5 days after the extra stress session (i.e. on day 10 after being subjected to physical or psychological stress for 5 days).

Surprisingly the data collected during this experiment was very similar to those obtained for Experiment 2, that is, 5 days stress and behavioural assessment 5 days later (Table 3.4 versus Table 3.2). Rats exposed to physical stress displayed significantly less rearing than psychologically stressed animals (Figure 3.16). Also no significant differences were observed in any of the behavioural parameters documented for the elevated-plus maze.

The addition of an extra stress session did not alter the secretion of corticosterone in any of the groups, hence no significant differences were observed in their plasma corticosterone concentrations (Figure 3.17).

TABLE 3.5: 5-DAY-STRESS, RESTRESS AND 10-DAY BEHAVIOUR

		Physical	Psychological	Control	p-value
	n = 26	9	9	8	
OPEN FIELD					
REARING	MEANS	4.001	9.429	7.025	**p ≤ 0.05**
	SEM	±0.959	±1.438	±1.932	
GROOMING	MEANS	0.778	1.111	0.375	p ≥ 0.05
	SEM	±0.364	±0.351	±0.263	
TIME IN INNER ZONE (sec)	MEANS	3.000	3.111	1.125	p ≥ 0.05
	SEM	±2.517	±1.327	±0.667	
TIME IN OUTER ZONE (sec)	MEANS	297.000	296.889	298.875	p ≥ 0.05
	SEM	±2.516	±1.327	±0.667	
NUMBER OF BLOCKS	MEANS	59.222	78.000	40.875	p ≥ 0.05
	SEM	±10.321	±11.641	±11.347	
ELEVATED PLUS					
REARING	MEANS	2.222	3.000	1.500	p ≥ 0.05
	SEM	±0.795	±0.601	±0.681	
GROOMING	MEANS	1.111	1.111	0.625	p ≥ 0.05
	SEM	±0.309	±0.539	±0.263	
TIME IN OPEN ARMS (sec)	MEANS	39.222	33.111	49.75	p ≥ 0.05
	SEM	±8.958	±7.467	±16.957	
TIME IN CLOSED ARMS (sec)	MEANS	260.778	266.889	250.25	p ≥ 0.05
	SEM	±8.958	±7.467	±16.957	
ENTRIES IN OPEN ARMS	MEANS	2.889	5.556	2.625	p ≥ 0.05
	SEM	±0.824	±1.180	±0.844	
ENTRIES IN CLOSED ARMS	MEANS	8.667	9.222	5.375	p ≥ 0.05
	SEM	±1.616	±0.909	±1.034	

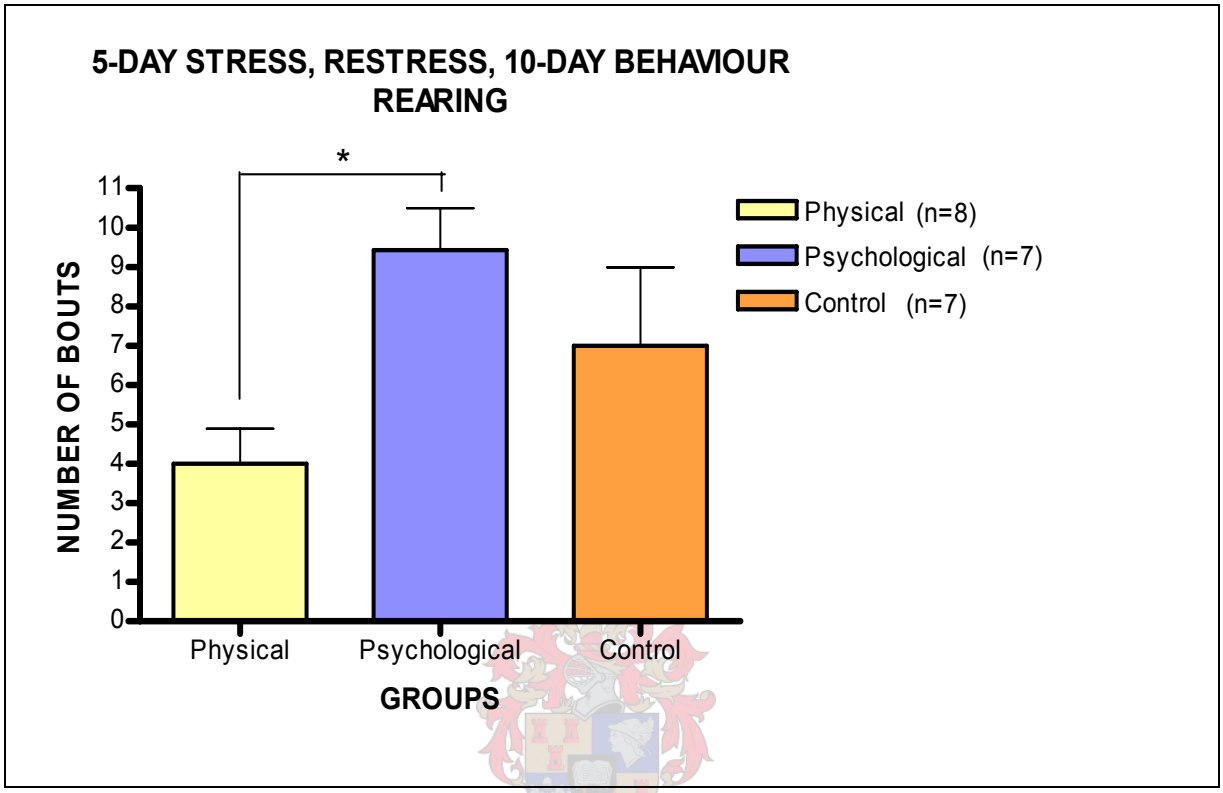


Figure 3.12. Amount of rearing in the open field. The animals were subjected to 5 day stress, re-stressed after 5 days and behaviour was assessed another 5 days later. Values are shown as means ±SEM. * $p \leq 0.05$; There was a significant difference between the physical and psychological groups.

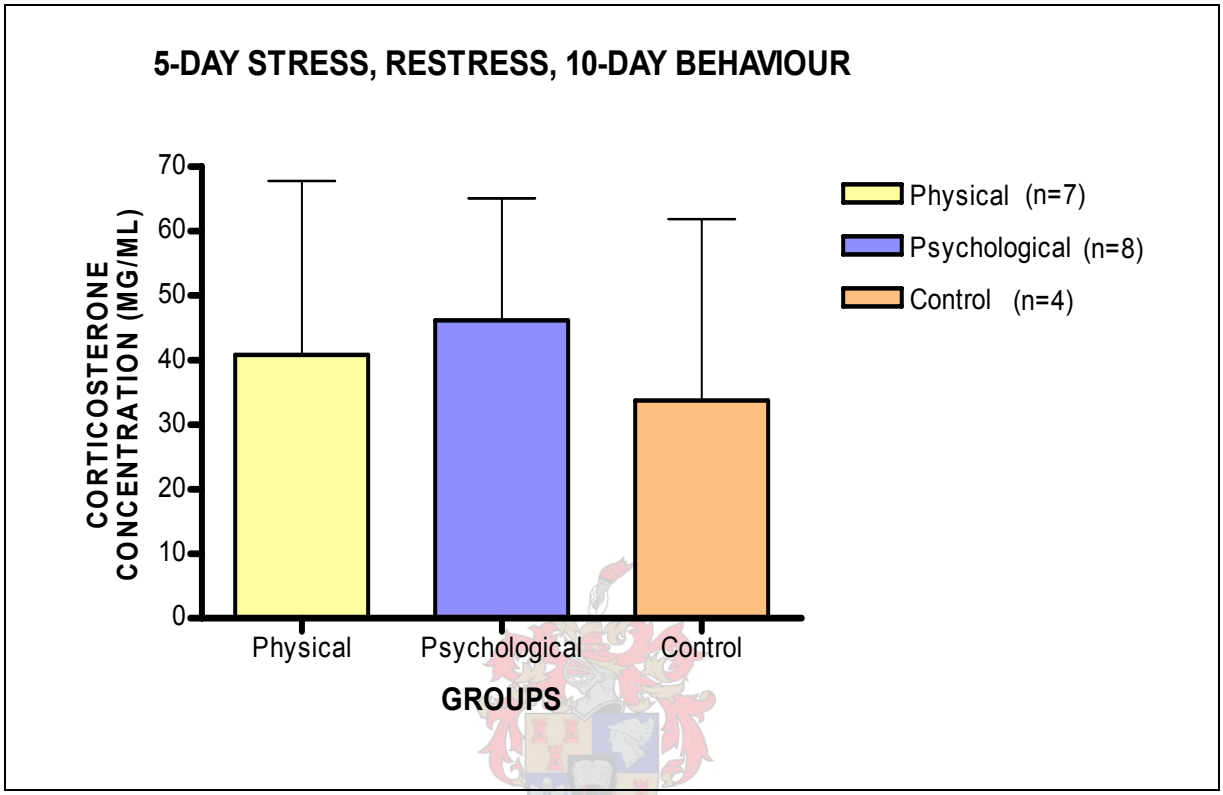


Figure 3.13. Corticosterone concentrations. The animals were subjected to 5 days stress, re-stressed after 5 days and behaviour was assessed another 5 days later. Values are shown as means ±SEM. There was no significant difference between any of the groups.

3.7. EXPERIMENT 6: (1-day stress physical and psychological, 4-day stress, 5-day behaviour)

A group of 26 rats was stressed for 5 days and on the first day of being stressed, both the physical and psychological groups were subjected to electric foot shock. This was done to familiarize the psychologically stressed animals with the actual physical stressor. In this experiment, the behavioural activity was again assessed 5 days after the last stress session.

Interestingly, significant results were obtained in both the open field and the elevated-plus maze tests (Table 3.3). Psychologically stressed rats spent significantly more time in inner zone, and significantly less time in the outer zone than the physically stressed rats (Figures 3.5 and 3.6) in the open field test. In addition, physically stressed rats showed a significantly reduced number of entries in the open arms in the elevated-plus maze (Figure 3.7), while psychologically stressed rats exhibited significantly more entries into the closed arms of the elevated-plus maze when compared to controls (Figure 3.8).

Measuring the corticosterone levels in these animals revealed (Figure 3.9) that no significant difference in plasma corticosterone levels existed between any of the groups.

TABLE 3.6: 1-DAY-STRESS PHYS AND PSYG, 4-DAY STRESS, 5-DAY BEHAVIOUR

		Physical	Psychological	Control	p-value
	n =26	11	11	4	
OPEN FIELD					
REARING	MEANS	7.545	12.000	5.750	P ≥ 0.05
	SEM	±2.090	±2.288	±1.797	
GROOMING	MEANS	2.000	1.182	0.75	P ≥ 0.05
	SEM	±0.426	±0.352	±0.250	
TIME IN INNER ZONE (sec)	MEANS	**0.818**	**15.182**	0.75	**P ≤ 0.01**
	SEM	**±0.553**	**±6.187**	±0.750	
TIME IN OUTER ZONE (sec)	MEANS	**299.182**	**284.818**	299.25	**P ≤ 0.01**
	SEM	**±0.553**	**±6.187**	±0.750	
NUMBER OF BLOCKS	MEANS	52.636	87.727	46.75	P ≥ 0.05
	SEM	±12.408	±16.129	±21.723	
ELEVATED PLUS					
REARING	MEANS	2.455	4.000	2.250	P ≥ 0.05
	SEM	±0.679	±0.726	±0.946	
GROOMING	MEANS	1.818	0.636	1.5	P ≥ 0.05
	SEM	±0.501	±0.203	±0.866	
TIME IN OPEN ARMS (sec)	MEANS	20.545	37.455	46.5	P ≥ 0.05
	SEM	±7.891	±9.926	±8.578	
TIME IN CLOSED ARMS (sec)	MEANS	269.909	240.273	245.25	P ≥ 0.05
	SEM	±10.585	±12.767	±9.656	
ENTRIES IN OPEN ARMS	MEANS	**0.909**	**3.727**	**5.75**	**P ≤ 0.001**
	SEM	**±0.251**	**±0.488**	**±0.854**	
ENTRIES IN CLOSED ARMS	MEANS	9.636	**12.727**	**9.000**	**P ≤ 0.05**
	SEM	±1.177	**±1.477**	**±0.707**	

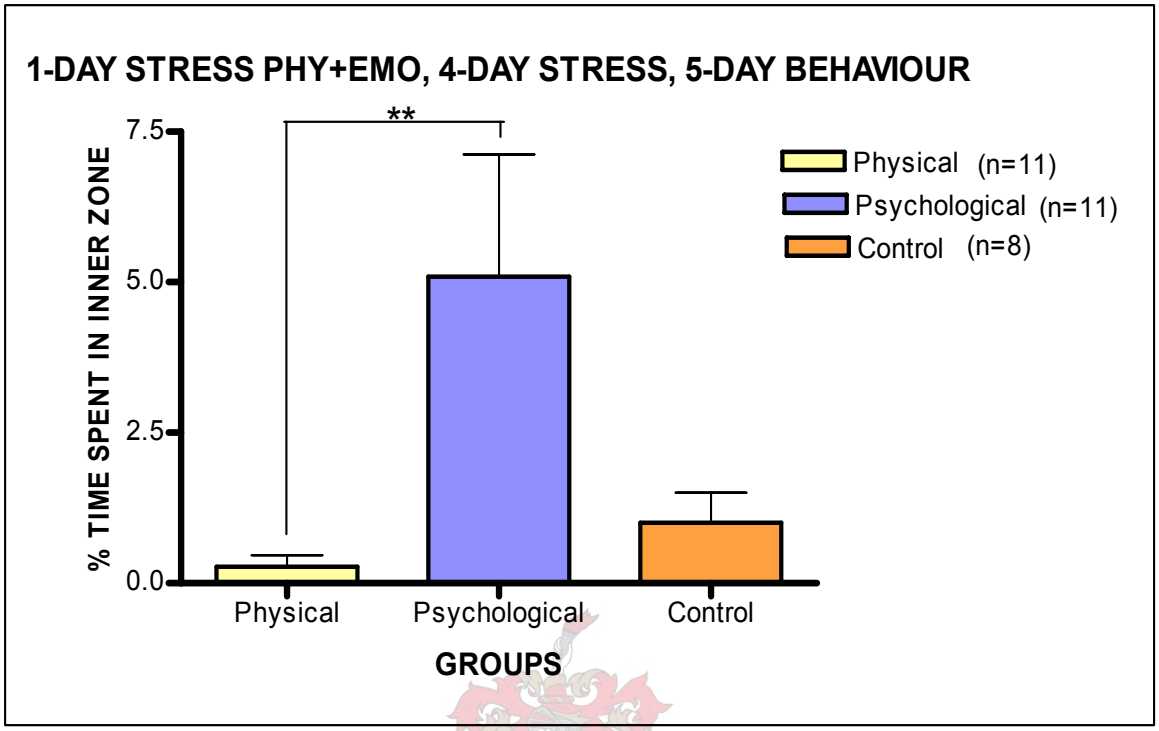


Figure 3.14. Percentage time spent in the inner zone of the open field. The physical and psychological animals were both stressed on day 1, from day 2 until day 5 only the physical group were stressed, and 5 days after that, behaviour was assessed. Values are shown as means ±SEM. ** $p \leq 0.01$; There was a significant difference between the physical versus the psychological stressed group.

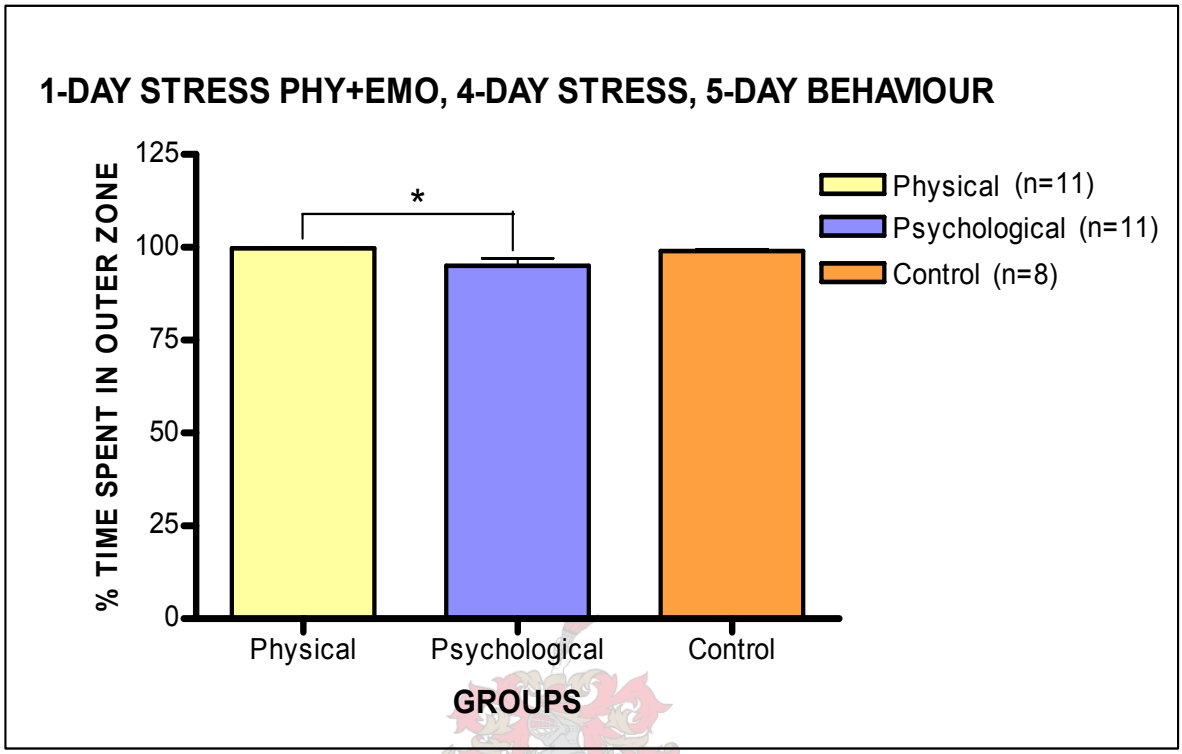


Figure 3.15. Percentage time spent in the outer zone of the open field. The physical and psychological animals were both foot shocked on day 1, from day 2 until day 5 only the physical group were stressed, and 5 days after that, behaviour was assessed. Values are shown as means ± SEM. * $p \leq 0.01$; There was a significant difference between the physical versus the psychological stressed group.

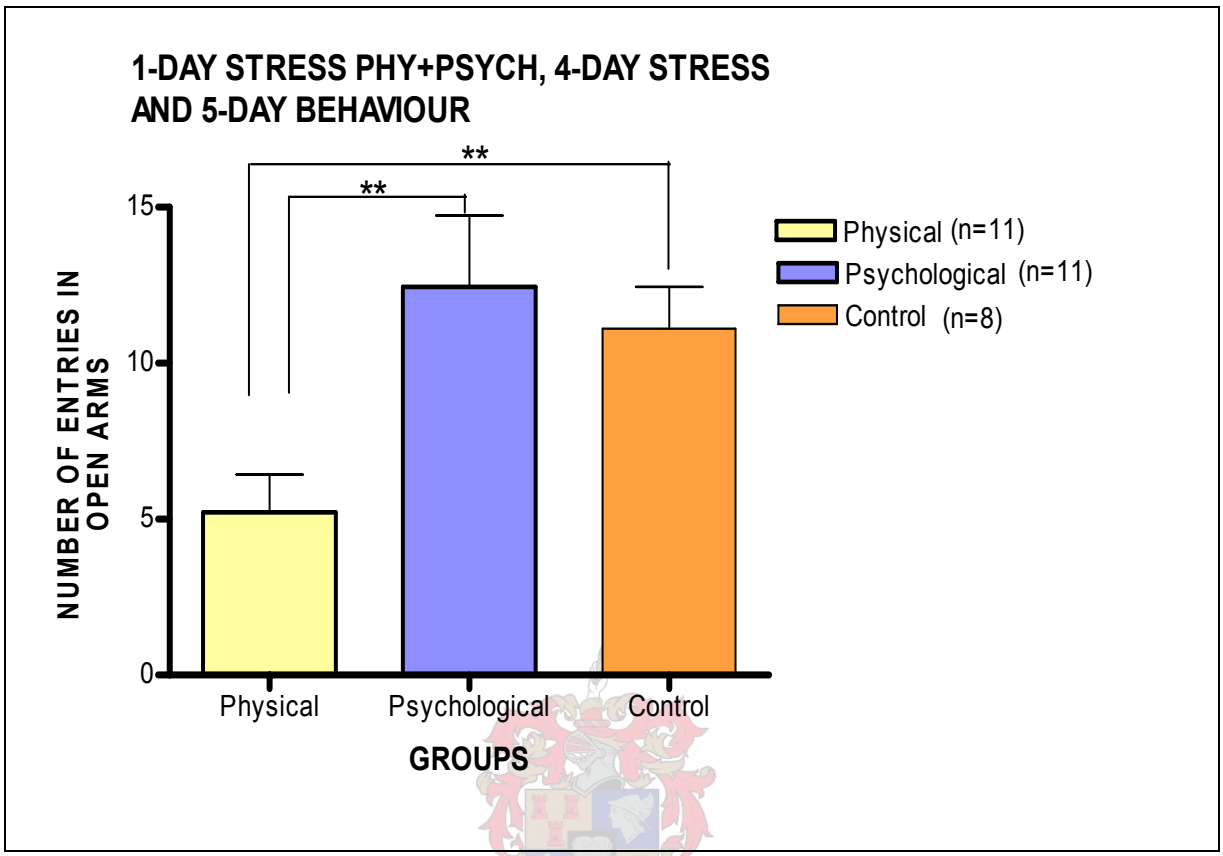


Figure 3.16. Number of entries in the open arms of the elevated plus-maze. The animals were stressed for 5 days and on the first day of being stressed, both the physical and psychological groups were subjected to electric foot shock. Values are shown as means ±SEM. ** $p \leq 0.01$; There was a significant difference between the physical versus the psychological stressed group, as well as between the physical stressed and the control group.

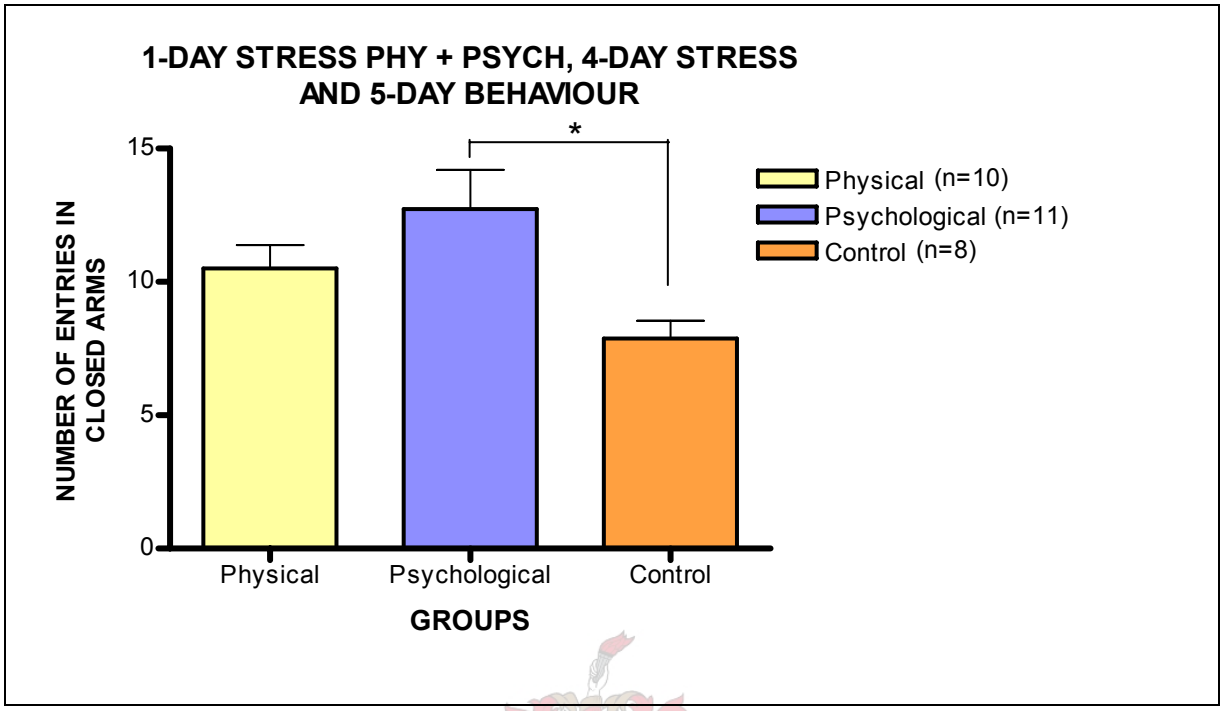


Figure 3.17. Number of entries in the closed arms of the elevated plus-maze. The rats were stressed for 5 days and on the first day of being stressed, both the physical and psychological groups were subjected to electric foot shock. Values are shown as means ± SEM. * $p \leq 0.05$; There was a significant difference between the psychological stressed and the control group.

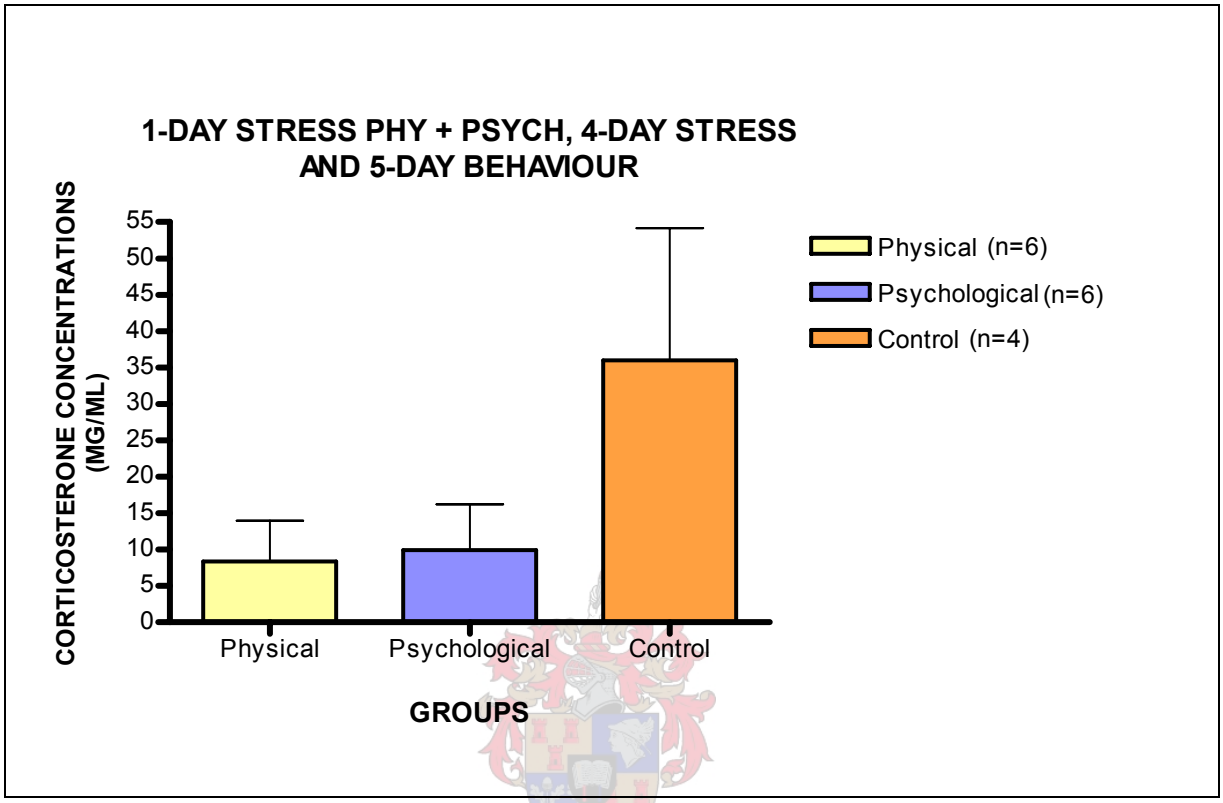


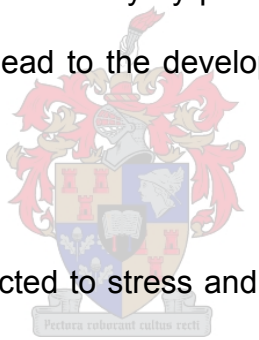
Figure 3.18. Corticosterone concentrations of animals that were stressed for 5 days and on the first day of being stressed, both the physical and psychological groups were subjected to electric foot shock. Values are shown as means ± SEM. There was no significant difference between any of the groups.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1. INTRODUCTION

Stress is considered one of the major factors involved in the pathogenesis of affective disorders (McEwen, 2000). According to Jedema (2002), behavioural alterations following chronic stress exposure, have been reported which are reminiscent of the alterations observed in patients afflicted with mood and anxiety disorders. It has been emphasized that the same biological and hormonal response to stress that protects the body by promoting physiological adaptation in the short run, can in fact also lead to the development of disease in the long run (Yehuda and McEwen, 2004).



Individuals are frequently subjected to stress and these stressors may be physical and/or psychological in nature. For instance, women and children are often subjected to abuse and assault, and other forms of physical stress such as violent crime are escalating daily (Jewkes *et al.*, 2005). Examples of psychological stressors include indirect exposure to terrorist attacks (Blanchard *et al*., 2005; Stein *et al*., 2004; Schuster *et al.,* 2001), perceptions of lack of control of a stressful situation (Ozer *et al*., 2003) or being subjected to subtle victimization (Estevez *et al.*, 2005). While it is well known that all these situations contribute to the development of psychopathology, the neurobiological mechanisms that precipitate the associated pathology remain unclear. The present study is therefore an

attempt to establish a model that may differentiate between physical and psychological stress, focusing on behavioural measures and neuro-endocrine responses.

4.2. THE EFFECTS OF PHYSICAL AND PSYCHOLOGICAL STRESS ON BEHAVIOUR

In order to characterise our model we have subjected rats to a number of stress paradigms (varying in duration), and assessed the effects thereof on behaviour at two different time points. Physically stressed rats were subjected to 10 unpredicted electric foot shocks (0.5 mille Ampere), in 10 minutes, while witnessing the application of the physical stressor, served as the psychological stressor. The experimental strategies are summarized in Table 4.1.

Two tests were employed to assess the behaviour of the animals, namely the Open Field test and the Elevated-Plus maze. One of the most widely used tests in behavioral research to evaluate environmental manipulations on the emotionality of rodents, is the open field test (Campbell *et al*., 2003; Prut and Belzung, 2003; Yehuda and Antelman, 1993). The apparatus consists of a large arena in which the animal is placed for a fixed amount of time, without having the chance to escape, since a wall surrounds the area. The floor is marked with lines to allow the quantification of locomotor activity. Open-field methods also allow for easy quantification of other aspects of locomotor behaviour that include exploratory behaviour, curiosity and coping. Therefore, behavioural parameters

such as grooming, rearing, and time spent in the inner and outer zone were noted. Animals were handled prior to the experiment to familiarize them with the apparatus and hence minimize the compounding effects of a novel environment.

Behavioral studies (Pijlman and Van Ree, 2002; Van den Buuse *et al.,* 2001) have shown rats to display higher avoidance and lower exploratory behaviour in open elevated alleys than in enclosed alleys. The avoidance of the open alleys is interpreted as being generated by the fear of novelty. The combination of height, luminosity and open space is assumed to induce fear or anxiety in the rat. This observation served as a basis for the development of the Elevated-plus maze. It is an apparatus with four elevated arms, 50 centimetres long and 10 centimetre wide, arranged in a cross. The two opposite arms are being enclosed and two open, having at their intersection a central platform that gives access to any of the four arms. Locomotor activity is indicated by the total number of arm entries. Other behaviours that are assessed include rearing, grooming and time spent in the open or the closed arms.

The open field and elevated plus maze tests have been thoroughly validated and subsequently widely used to determine locomotor activity and the level of anxious behaviour in rodents (Uys *et al*., 2003; Rodgers and Dalvi, 1997; Hogg, 1996; Yehuda and Antelman, 1993; Corum and Thurmond, 1977). It is postulated that “avoidance-escape deficit” following certain highly stressful conditions result from changes in activity of noradrenergic or other mono-aminergic neural systems.

TABLE 4.1: SUMMARY OF EXPERIMENTS	n	M	T	W	T	F	S	S	M	T	W	T	F	S	S	M	T
DAYS		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1-day stress, 5-day behaviour	26					↯					Beh	Dec					
5-day stress, 5-day behaviour	30	↯	↯	↯	↯	↯					Beh	Dec					
5-day stress, 5-days - restrain, 15min	22	↯	↯	↯	↯	↯					Re/Dec						
Decapitation																	
5-day stress, 5-days - restrain, 60min	20	↯	↯	↯	↯	↯					Re/Dec						
Decapitation																	
5-day stress, 10-day behaviour	26	↯	↯	↯	↯	↯										Beh	Dec
5-day stress, 10-day behaviour, 10 days	30	↯	↯	↯	↯	↯										Beh	Dec
Citalopram treatment																	
5-day stress, restress and 10-day behaviour	26	↯	↯	↯	↯	↯					↯					Beh	Dec
1-day stress phys and psych, 4-day stress,	26	↯	↯	↯	↯	↯					Beh	Dec					
5-day behaviour																	
TOTAL	206																

Received foot shock (0,5mA), randomly 10 times in 10 minutes	↯
Behaviour in open field and elevate-plus maze	Beh
Decapitation and blood collection	Dec
Restraint rat for 10 min	Re
Treatment with Citalopram	

Therefore, the expression of many behavioural patterns is stress-sensitive in that their expression may be secondary to alterations in neurotransmitter or hormone function. For instance, the actions of stress hormones may be constrained by the activity of other hormones as well as by environmental circumstances (Greenberg *et al.,* 2002). Another consideration that needs to be kept in mind is that the lack of effects on some behavioural tests may result from the stressfulness of the test itself and therefore the interpretation of behavioural data should always be done with caution (Pijlman and Van Ree, 2002).

Our experiments (Table 4.2) showed remarkable differences in a number of behavioural measures:

1. The behavioural outcomes of animals, as determined **5 days** after being subjected to a **single session of foot shock**, delivered significant results. Rats exposed to physical stress displayed significantly more grooming, in comparison to psychologically stressed and control rats.
2. Rats that were exposed to **repeated stress for 5 days** and assessed **5 days later**, exhibited a totally different picture. Here physically stressed animals showed significantly less rearing, when compared to psychologically stressed rats and controls. No significant differences were observed between the latter two groups.
3. When the behaviour of rats that received **5 days of repeated stress**, were assessed **10 days** after the last stress session, both groups of stressed animals exhibited behavioural abnormalities. Both physically and

psychologically stressed rats displayed reductions in rearing and elevated levels of anxiety. In addition, physically stressed rats also showed significantly decreased locomotor activity.

TABLE 4.2: SUMMARY OF BEHAVIOURAL DATA

EXPERIMENTS	REARING		GROOMING		LOCOMOTION		ANXIETY	
	PHYS	PSYCH	PHYS	PSYCH	PHYS	PSYCH	PHYS	PSYCH
1-DAY STRESS			↑					
5-DAY STRESS	↓							
5-DAY STRESS-10-DAY BEHAV	↓↓	↓			↓		↑↑	↑
CITALOPRAM TREATMENT					↓			
RESTRESS	↓							
5-DAY STRESS, 1-DAY PHYS/PSYC		⇧				⇧		↑

SIGNIFICANTLY MORE INCREASED COMPARED TO CONTROL	↑
SIGNIFICANTLY MORE DECREASED COMPARED TO CONTROL	↓
A TENDENCY TO BE INCREASED	⇧

These findings are interesting in a number of ways. Firstly, they indicate that exposure to a single session of stress can lead to behavioural abnormalities depending on the type of stress. Structural and temporal characteristics of behaviour may provide clues as to the organizational and motivational principles that guide co-coordinated behaviour. For example, a behaviour that occurs naturally has a diverse, patterned structure, particularly when evoked under

predictable conditions. Grooming in rats meets these criteria. Physically stressed rats, in the single stress experiment, groomed excessively in comparison with the psychologically stressed rat as well as the control rats. Increased grooming is known to be triggered by environmental disturbance, such as conditions of stress that include handling, restraint and novelty (Kalueff and Tuohimaa, 2005; Komorowska and Pellis, 2004). While our results suggest that grooming may be directly related to the strength of the stressor, this may not necessarily be the case. According to a study done by Van Erp *et al*. (1994), rats used as intruders in the territory of another rat, maintain a constant low level of grooming. In contrast, rats placed in a novel cage steadily increased grooming during the observation period. These results indicate that grooming cannot be simply understood as an immediate response required to reduce the level of arousal following stressors. Instead, following exposure to a stressor, grooming may be seen as a self-soothing behaviour that assists in coping with the stressful situation.

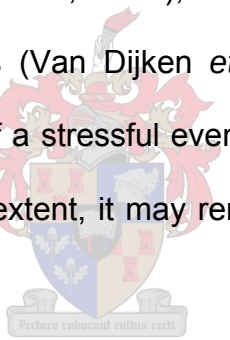
It has long been recognized that the interaction of grooming and anxiety is rather complex and that rat grooming can be increased in both high and low stress situations. For example, after anxiolytic and anxiogenic drugs, both activation and inhibition of grooming was observed (Pellow *et al.,* 1985b). Grooming activity is therefore also considered a mechanism serving to alleviate anxiety, i.e. as a displacement activity in rats. It is well known that animals often make extensive use of displacement activity, such as freezing behaviour and/or jumping, as a strategy to cope with stress (Kualeff and Tuohimaa, 2005).

Many behavioural variables on their own however are poor markers of stress and stress-related states (Komorowska and Pellis, 2004). Therefore, combining a number of behavioural parameters generates a better impression as to the behavioural status of an animal. In view of this, we subjected the rats to repeated bouts of stress in the hope that its impact on their behaviour would result in more pronounced symptoms.

A second interesting finding therefore was the substantial difference in behaviour (again assessed 5 days after the last stress session) obtained when the rats were exposed to repeated stress as opposed to a single bout of stress. The physically stressed rats showed reduced rates of rearing, while the psychologically stressed rats showed similar levels to the control animals. Low levels of rearing in the open field test may reflect high emotionality and fearfulness (Van den Berg *et al.,* 1998), and anxious behaviour (Carneiro *et al.,* 2005). It seems therefore that our animals that were exposed to physical stress definitely displayed some sign of anxious behaviour. Rearing is a component of exploratory behaviour and when displayed in an open field, the behaviour may be multi-factorial. With exploration and stress being the main motivational systems underlying rearing behaviour (Crusio and Schwegler, 2005), variations observed in its expression may therefore be due to differences in either of these two factors. Increases in rearing behaviour may also reflect other advantages. When animals are under threat or are attracted by novel stimuli, increased exploration may in fact improve the chances of finding life necessities (food) or promote survival (finding shelter or escape routes).

Similar to grooming, interpretation of rearing data alone may be problematic. In our search to obtain a better understanding of the effects of physical stress versus psychological stress, rats were subjected to stress repeatedly for 5 days and assessed 10 days later. At this time point both physically stressed and psychologically stressed rats showed significant behavioural abnormalities. Both groups of animals had reduced rates of rearing and elevated levels of anxiety. These observations were comparable to experiments where uncontrollable electric foot shocks caused decreased swimming activity in mice (Prince and Anisman, 1984). In the elevated plus maze the rat's behaviour is largely based on its anxiety level when placed in the apparatus for the first time (Giddings, 2001). The animal's preference for the closed arms over the less secure open arms is used as a parameter to indicate the level of anxious behaviour. More support for our findings came from studies by Estanislau and Morato (2005), where rats that were exposed to indirect in-utero stress (their dams were subjected to electric foot shock) displayed a reduction in exploration of the open arms of an elevated plus maze. In another experiment Van der Hart *et al.* (2005) demonstrated how tree shrews, subjected to a 7 day period of psychosocial stress, also displayed decreased locomotor activity. These studies clearly show an association between prolonged stress, decreased locomotor activity and an increased state of anxiety, and our results strongly support this concept in both the physical and psychologically stressed groups.

Furthermore, our findings reflect a development of behavioural deficits over time. There was a clear distinction between the observed behavioural abnormalities in animals when assessed 10 days rather than 5 days after stress exposure. This phenomenon was evident in both physically and psychologically stressed groups and suggested the development of a long-term facilitation process in the brain such as sensitization or habituation. In these processes the molecular changes that may be involved in the transition to forms of memories of longer duration, depend not only on the intensity of the stimuli, but also on the time of application. Interestingly exposure to foot shock stress, as a predictable event, induced long-lasting memories for 96 hours (Jodar *et al*., 1994), but as an unpredictable event, its effects may last up to 4 weeks (Van Dijken *et al.,* 1992). These observations therefore show that the impact of a stressful event may require time to evolve and once it has developed to its full extent, it may remain present and consistent over an extended period of time.



The notion of the development and then persistence of abnormal behaviour following an adverse event is strongly supported by clinical studies. For instance, some studies have provided evidence showing adverse experiences during childhood to be a major contributory factor to psychopathology later in life. For example, sexually abused girls developed depression and/or anxiety-related disorders as adults (Heim *et al.*, 2000; De Bellis *et al.*, 1994), while post-traumatic stress disorder in war veterans has been diagnosed long after the termination of the battle (Hilsenroth *et a*l., 2005). In these human studies the serotonergic system

has been strongly implicated in mediating the adverse effects of stress in precipitating the mentioned psychopathologies (Heim and Nemeroff, 2001; Kaufman and Charney, 2000), and as such, many patients have been treated successfully with drugs that modulate this monoaminergic system in the brain. In view of this, we have investigated the efficacy of citalopram, a selective serotonin re-uptake inhibitor, to reverse the behavioural deficits of physically and psychologically stressed rats. In this experiment, rats were treated with a daily dose of Citalopram (10 milligram/kilogram, intra peritoneal.) for 10 days, after being subjected to 5 days of stress.

Citalopram exhibited a classical antidepressant-like effect by normalizing the reduction rearing and high level of anxiety in both groups. Interestingly, locomotor activity in the physically stressed rats remained unchanged. High levels of anxiety (including changes in rearing and locomotor activity) induced by early separation stress have been proposed to stem from decreased noradrenergic and increased serotonergic function (Daniels *et al*., 2004). It was therefore not surprising that citalopram was indeed able to reverse parameters of anxiety and rearing. This positive response in both groups of animals demonstrated good predictive validation and indicated a usefulness of the models for antidepressant evaluation.

The brain processes involved in locomotor activity are regulated by the mesolimbic dopamine system. Therefore alterations in locomotor activity may be attributed to a disturbed dopamine system (Dunn, 2000; Koolhaas *et al.,* 1999), and hence the

inability of citalopram to restore this parameter. It is clear that more than one neurotransmitter system is involved in precipitating stress-induced behavioural abnormalities and therefore effective antidepressant therapy must perhaps focus on targeting multiple neurotransmitters. An alternative explanation is that citalopram in our study was just ineffective in restoring locomotor activity in Sprague Dawley rats, because in an experiment done by Millan and co-workers, they demonstrated that selective serotonin reuptake inhibitors, such as citalopram, enhance locomotor activity only in mice (NMRI strains) exposed to a novel environment but not Sprague Dawley and Wistar rats (Millan *et al*., 1998).

Relief from behavioural changes often indicates the development of stress adaptation, which may entail hypersensitivity of the serotonin system after repeated stress (Ohi *et al.,* 1989). However, hypersensitivity was not observed after exposure for 5 days and therefore this reasoning may be unlikely in our experiments. It is therefore obvious that further experiments are required to delineate how the serotonergic and other neurotransmitter systems orchestrate abnormal behaviour following different types of stress.

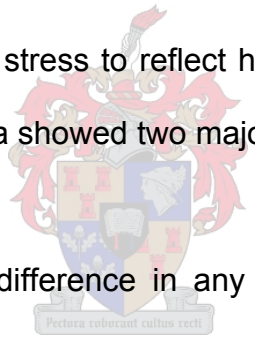
To further characterize our model, another group of rats was stressed for 5 days and then subjected to an additional stress session 5 days after the last stress. The behaviour assessment was done 5 days after this re-stress session, i.e. 10 days after being subjected to the 5 day physical or psychological stress protocol. The physically stressed animals showed significantly less rearing, while psychologically

stressed rats and controls had similar bouts of rearing. This result was similar to that obtained when the animals were just exposed to 5 days of stress and assessed 5 days later. Exposure for the second time (re-stress) to the same test situation did therefore not worsen the behavioural deficits seen in the previous experiment. Instead, this observation supported our reasoning that behavioural abnormalities develop over time as comparable data was obtained in both experiments. We would therefore predict that the behavioural measurements, if collected 10 days after the re-stress, would include alterations in locomotor activity and level of anxiety in both groups of animals.

We designed another experiment where both the physically and psychologically stressed groups were subjected to electric foot shock on the first day. After this initial stress session, the two groups were again handled as previously described, i.e. physically stressed animals receiving electric foot shocks for the remainder of the 5 day stress period, while psychologically stressed animals had to witness this event. The psychologically stressed animals were therefore familiar with the actual physical stressor. Behavioural activity was again assessed 5 days after the last stress session. Totally unexpected results were obtained. The psychologically stressed animals displayed markedly increased exploratory behaviour. The animals also showed a tendency to be more active and exhibit greater rearing than the other two groups of animals. Elevations in these parameters may indicate a state of hyperarousal and therefore present with activities that reflect strategies to avoid shock exposure.

4.3. THE EFFECTS OF PHYSICAL AND PSYCHOLOGICAL STRESS ON NEURO-ENDOCRINE RESPONSE

One of the dominant parts of the stress response is neuro-endocrine activation. Therefore studying the functioning of the hypothalamic-pituitary-adrenal axis is of importance in our quest to characterize physical and psychological stress. Two approaches were adopted in the present experiments to provide us with information about the effects of physical and psychological stress on the hypothalamic-pituitary-adrenal axis: 1) baseline corticosterone concentrations were determined to indicate basal hypothalamic-pituitary-adrenal axis activity, and 2) rats were subjected to acute restraint stress and the corticosterone concentrations were measured at time points 15 and 60 minutes post stress to reflect hypothalamic-pituitary-adrenal axis responsivity. Basically, our data showed two major findings:



1. there was no significant difference in any of the groups at any time point studied with respect to their basal plasma corticosterone levels,
2. both physically and psychologically stressed rats displayed a blunting of the stress response following acute restraint stress.

The fact that there was not significant differences in baseline corticosterone concentrations between any of the groups of animals, suggest that the normal circadian activity of the hypothalamic-pituitary-adrenal axis remained intact despite various stress protocols. This is not surprising, as a number of basic studies have reported normal basal corticosterone levels in rats exposed to electric foot shock

stress. For instance, in two recent studies, rats were subjected repeatedly to inescapable foot shock and these authors also observed no changes in baseline corticosterone levels (Louvart *et al*., 2005; Helmreich *et al*., 2005). Even in clinical studies, while many reported psychiatric disorders are associated with abnormalities in hypothalamic-pituitary-adrenal axis regulation (Otte *et al.,* 2005), others showed no difference in basal cortisol concentrations between patients and controls (Burke *et al*., 2005; Bachmann *et al*., 2005). It is obvious that basal plasma glucocorticoid levels as a parameter of hypothalamic-pituitary-adrenal axis activity remain controversial and should therefore be investigated more.

In contrast to the basal concentrations, both physically stressed and psychologically stressed animals showed diminished levels of corticosterone 15 minutes after the acute restraint stress. This reduced corticosterone secretion indicated a blunting of hypothalamic-pituitary-adrenal axis responsivity to mediators of stress. This result is in accordance with earlier studies from our laboratory where early postnatal maternal separation also led to a hypo-responsive hypothalamic-pituitary-adrenal axis (Daniels *et al*., 2004). Interestingly Pijlman (2001) showed physically and psychologically stressed rats to have a differential endocrine response. Physically stressed animals showed increased corticosterone levels compared to controls, while psychologically stressed animals had the same stress response as the control animals. Discrepancies in this data and ours may derive from differences in the respective experimental protocols. These include the use of different rat strains, variations in current strength and timing of application during

the foot shocks, as well as dissimilarities in time after stress exposure when blood was collected for the determination of hormone concentrations.

According to McEwen (2000), acute stress is frequently followed by an activation of a delayed hypersensitivity response and this is related to the magnitude of glucocorticoids secretion. Chronic stress, on the other hand, produces a suppression of the delayed hypersensitivity and also suppresses the initial sensitization of the response. Such a suppressive effect may cause the blunting hypothalamic-pituitary-adrenal axis response observed in our experiments. In support of this kind of reasoning is Yehuda and Antelman's hypothesis (1993) that the stress-restress paradigm may produce sensitization in some biological systems, in this case, inhibitory elements of the hypothalamic-pituitary-adrenal axis, to yield abnormal corticosterone secretion. Upregulation of hippocampal glucocorticoid receptors that function to inhibit hypothalamic-pituitary-adrenal axis activity, may therefore be involved (Liberzon *et al*.,1997).

Experiments by Johnson *et al.* (1992) showed that during constant stress, the effectors of the generalized stress response (including the hypothalamic-pituitary-adrenal axis) interfered with, rather than promote the animal's ability to adapt to the stressful situation. However, unpredictability of the stressor seemed to be a critical factor in this inability to habituate. In addition, in animal models of chronic stress, the acute stress response often displays signs of habituation when the stressor is presented repeatedly (Jodar *et al*., 1994). It may therefore be possible that the

random delivery of foot shocks used in our experimental repeated stress paradigm could have partially prevented similar adaptive responses, and hence no changes in baseline corticosterone levels but significant differences in hypothalamic-pituitary-adrenal axis response to restraint stress.

Plasma corticosterone inhibits corticosterone-releasing-factor and adreno-corticotropic-hormone secretion under normal conditions of acute stress. Under chronic stress, this negative feedback inhibitory effect may have been amplified resulting in a down-regulation of corticosterone-releasing-factor and adreno-corticotropic-hormone release upon acute stress. This explanation is plausible as patients suffering from post-traumatic stress syndrome exhibit a hypersensitivity of the hypothalamic-pituitary-adrenal axis to cortisol feedback inhibition (Yehuda and Antelman, 1993). According to Van Dijken *et al.,* (1992) increased release of vasopressin may underlie the sensitization of the hypothalamic-pituitary-adrenal axis, since vasopressin stores were found to be increased in the external stores of the median eminence where fibers of the corticotropin-releasing-hormone neurons are located. Stressful stimuli are known to specifically upregulate vasopressin Ribonucleic acid in these corticotropin-releasing-hormones and vasopressin can potentiate the corticotropin-releasing-hormone induced release of adreno-corticotropic-hormone in the pituitary (Van Dijken *et al.,* 1992). This hypothesis was tested by Schmidt *et al*. (1996) who investigated the long-term changes in vasopressin following the administration of interleukin-1β to rats. These researchers reported that a single injection of interleukin-1β sensitized the adreno-

corticotropic-hormone and corticosterone response 11 days later and induced a long-term increase in vasopressin stores in the median eminence. In contrast, exposure to two short episodes of social defeat did not up-regulate vasopressin but rather induced temporal dynamic changes in the hypothalamic-pituitary-adrenal axis indicative of increased feedback inhibition (Schmidt *et al*., 1996). These findings suggested that qualitative characteristics are also important determinants of the ability of a stressor to alter hypothalamic-pituitary-adrenal axis activity over time.

4.4. SUMMARY AND CONCLUSIONS

Original concepts of stress have undergone much revision in the last few decades. It is now clear that there are differences in both how organisms respond to different types of stressors, as well as how they respond to the same stressor. When faced with severe stress, humans are built to survive. While some individuals cope with extreme stress without psychopathological consequences, others develop severe psychiatric conditions such as posttraumatic stress syndrome or major depression. Chronic environmental stress (starting a new job, loss of close relative or divorce) or experiencing an adverse life event can also precipitate anxiety disorders, schizophrenia and depression. The response to severe stress may be adaptive in the short-term, but if the person affected does not reset their psychobiological "thermostat", then psychopathology ensues (Olsson, 1999).

The focus of the present study was to investigate behavioural and endocrine responses in rats subjected to physical and psychological stress. In summary, our data showed that

1. single stress exposure may lead to different outcomes as repetitive stress exposure,
2. the consequences of stress exposure develop over time and persist for an extended time period,
3. these consequences of direct stress exposure versus indirect stress exposure show a grading in stress intensity and perception, similar to that observed in humans,
4. citalopram is effective in reversing anxious-like behaviours, but not locomotor deficits,
5. basal plasma corticosterone concentrations were comparable in all the animals,
6. physically and psychologically stressed rats displayed a hyposensitive hypothalamic-pituitary-adrenal axis following acute restraint stress.

It was interesting to note that the same stress given for different time period (1 day versus 5 days) elicited different behavioural outcomes. This may reflect the animal's adaptive response to the stress i.e. utilizing a number of behavioural strategies to cope with the stress. This seemed plausible as the employment of complex behaviours (grooming → rearing → locomotor activity) increased as the chronicity of the stress increased. Similarly interesting was the fact that the

dysregulation of the hypothalamic-pituitary-adrenal axis was evident in both physical and psychologically stressed animals, suggesting the sensitivity of this neuroendocrine system to stress.

These observations are in line with human studies showing that indirect exposure to stress results in symptoms that can be described as a milder form of those symptoms elicited by direct exposure. It is well known that the effects of stress are not always detrimental, as its by-products (learning, growth and adaptation) are a necessary part of life (Greenberg *et al.,* 2002). However, prolonged, recurrent stress can present insurmountable obstacles and yield maladaptive consequences (Yehuda and McEwen, 2004). For example, Post-traumatic stress syndrome and depression are but two disorders associated with repetitive traumatic experiences and dysregulated glucocorticoid secretion. Our stress models therefore propose to be useful in elucidating the complex interrelationship between an external event or stressor, and the organism experiencing it. Simultaneously it presents a promising platform for the finding of new or better treatments for patients.

4.5. FUTURE RESEARCH

While illuminating many aspects of the stress response mechanisms and opening the door for therapeutic interventions, recent research on the topic has also raised some questions that may form the basis of future studies. For instance, corticosteroids and serotonin are both linked to anxiety disorders and depression (Helmeke *et al.,* 2001); however the exact mechanisms and the functional

characteristics of the cross talk between corticosteroids and serotonin are largely unknown. It is therefore necessary to investigate the signal transduction pathways that are activated, which genes, proteins and other factors are involved in the dysregulation of the serotonin system by the neuro-endocrine system.

One problem with research into the physiology of stress is that the preponderance of such research has been conducted in males. Prior to 1995, females constituted only about 17% of participants in laboratory studies of physiological and neuro-endocrine responses to stress, while in recent years, the gender bias has somewhat decreased. Women are reported to have a higher susceptibility to stress related psychiatric illnesses and women constitute the majority of patients receiving antidepressant treatment. It is important to remember that a female may act differently in stressful situations to a male. These assumptions are based on the fact that stress effects on dendritic atrophy occur in males but not in females. There was evidence of neuronal damage in male rats undergoing cold-swim stress that was not evident in females (McEwen, 2000). The question therefore arises: How do gender differences in the response to stressful challenges influence the brain's response in terms of resilience or damage? Could the differences in mechanisms play a role in the fact that men are more likely to die of chronic disease of the cardiovascular system, while women appear more likely to suffer from a wide range of functional disorders?

Other emerging questions include - how can we apply our knowledge of the neural circuitry involved in fear responses to the results of functional neuroanatomical studies? What are the conditions that determine whether brain structures will respond with adaptive plasticity or show permanent damage? How does the vulnerability to permanent damage change in the aging brain? How can posttraumatic stress syndrome be associated with both hypersecretion of corticotropin releasing hormone and low levels of circulating cortisol? The beauty of the present study resides in the fact that attempts can now be made to address the above-mentioned questions under controlled laboratory conditions. And by doing so, contribute meaningfully to our current understanding of the psychobiology of stress and stress-related disorders.

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