

**THE ROLE OF MONOAMINES IN POST TRAUMATIC
STRESS DISORDER (PTSD) USING A TIME DEPENDENT
SENSITIZATION ANIMAL MODEL**

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This study is dedicated to.....

All the victims of suffering

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

**By (the Token of) Time (through the ages), Verily Man is in
loss,**

**Except such as have Faith, and do righteous deeds, and
(join together) in the mutual teaching of Truth, and of
Patience and Constancy
(The Holy Quraan 103:1-3)**

Abstract

Posttraumatic stress disorder (PTSD) is an anxiety disorder that may result from an exposure to a severely traumatic life-event. It is characterised by a delayed onset of psychological and physical symptoms including re-experiencing the event, avoidance of reminders associated with the trauma, increased autonomic arousal and distinct memory deficits. This disorder is also characterised by a maladaptive hypothalamic-pituitary-adrenal (HPA)-axis response and altered monoamine concentrations in the hippocampus and pre-frontal cortex.

The **Time Dependent Sensitization (TDS)** model is a putative animal model of PTSD that is based on the concept of repeated trauma, using **three acute stressors (TS)** of intense severity followed by a mild **situational reminder (RS)** on day 7 subsequent to the acute stressors. The aims of this study were to determine if the Triple Stressor (TS) induces stress and if the situational reminder (RS) is necessary for the maintenance of the stress response over time and whether these two stress responses are qualitatively and quantitatively different. This was done to further validate the TDS model and to characterize the development and progression of the stress-related pathology of PTSD.

Methods used were **High Performance Liquid Chromatography (HPLC)** with electrochemical detection (biochemical correlates) for quantifying the monoamines dopamine (DA), noradrenaline (NA) and serotonin (5-HT) concentrations in the hippocampus and pre-frontal cortex (PFC); **radio immuno assay (RIA)** for the determination of plasma corticosterone concentrations (neuroendocrine parameter) and the use of the **Elevated Plus Maze (EPM)** to detect anxiety-like behaviour (behavioral analyses).

The study was sub-divided into an Acute and Re-Stress study ($n = 10$). In the **Acute Study** rats were exposed to TS as the only stressor. Group 1 was sacrificed immediately after TS, Group 2 was sacrificed 3 days post TS and Group 3 on day 7 post TS. In the **Re-Stress Study** both TS and RS were used as stressors. Group 4 was sacrificed immediately after the situational reminder, Group 5 was sacrificed 3 days post RS and Group 6 on day 7 post RS. A group of unstressed rats were used as Control.

The results of this study found corticosterone concentrations elevated immediately after the TS ($p<0.05$). Exposure to the RS resulted in a profound hypocortisolism ($p<0.05$). These results indicate a possible disturbance in the regulation of the HPA-axis, which manifests as an enhanced negative feed-back upon re-introduction of the stressful situation.

Changes in MA concentrations were evident. Although no definite fixed trend is apparent in this study, it is evident that the TDS model does induce monoamine dysregulation. Hippocampal NA, DA and 5-HT concentrations were noted to be elevated on day 7 post TS ($p<0.05$). On day 7 post RS only hippocampal 5-HT was decreased significantly ($p<0.05$).

Behavioural analyses indicate that stress related anxiety was not sustained after the TS but 7 days after the exposure to the RS rats were most anxious ($p<0.05$). The results confirm that the TDS model does induce PTSD-like symptoms in rats and that the situational reminder (RS) is necessary for the maintenance of the stress response. This model may be useful in the investigation of future experimental pharmacological interventions in the management of PTSD.

Key words: Posttraumatic stress disorder (PTSD), corticosterone, time dependent sensitisation (TDS) model, elevated plus-maze (EPM), hippocampus, pre-frontal cortex (PFC), monoamines, rats.

Opsomming

Posttraumatiese angs-steurnis (PTAS) is 'n angstoestand wat ontstaan na blootstelling aan 'n erge traumatiese ervaring. Dit word gekenmerk deur 'n vertraagde aanvang van psigologiese en fisiese simptome wat aanhoudende herlewering van die trauma, vermyding van herinneringe verbonde aan die trauma, verhoogde outonadiese opwekking, en definitiewe geheueverlies insluit. Dit word verder gekenmerk deur 'n ingeperkte hipotalamus-pituitêre-adrenale (HPA)-as reaksie en veranderinge in die monoamienkonsentrasies in die hippokampus and pre-frontale korteks.

Die **Tyd-Afhanklike Sensitisasie (TAS)** model is 'n eksperimentele dieremodel van PTAS wat gebaseer is op die konsep van herhaalde trauma. Die doelwitte van hierdie studie was om te bepaal of die **Drievoudige Stressor (DS)**, stres induseer en of die **herstressor (HS)** noodsaaklik is vir die instandhouding van die stres respons oor tyd, asook om te bepaal of hierdie twee stres reaksies kwantitatief verskil. Dit is gedoen met die oog daarop om die TAS model verder te valideer en om die verloop van stres-verwante patologie van PTAS te karakteriseer.

Metodes wat gebruik is, sluit in **hoëdigtheidsvloei-stofchromatografie (HDVC)** met elektrochemiese deteksie (biochemiese parameter) vir die kwantifisering van die monoamien (dopamien, noradrenalin en serotonien) in die hippokampus en pre-frontale korteks (PFK) en **radio-immunologiese** bepalinge vir die vasstelling van plasmakortikosteroon konsentrasies (neuro-endokriene parameter). Die "**Elevated Plus Maze (EPM)**" is gebruik om angstige gedrag waar te neem (gedragsanalise).

Die studie was verdeel in 'n Akute Studie en 'n Herstres Studie ($n = 10$). Gedurende die **Akute Studie** is die rotte blootgestel aan die TS as die enigste stressor. Groep 1 is onmiddelik onthoof na die blootstelling, Groep 2 is 3 dae na blootstelling en Groep 3 op dag 7 na blootstelling onthoof. In die **Herstres Studie** is beide die TS and HS gebruik as stressors. Groep 4 is onmiddelik onthoof na die herstres, Groep 5 is 3 dae na HS en Groep 6 op dag 7 na HS onthoof. 'n Groep rotte wat nie gestres is nie, is as Kontrole groep gebruik.

Verhoogde kortikosteroonkonsentrasies is onmiddelik na die TS ($p < 0.05$) getoon. Blootstelling aan die HS het 'n definitiewe hipokortisolemie veroorsaak ($p < 0.05$). Hierdie resultate toon 'n

versteuring in die beheer van die HPA-as wat veroorsaak dat daar 'n verhoogde negatiewe terugvoer is met 'n daaropvolgende blootstelling aan 'n stresvolle situasie. Verskeie veranderinge in monoamienkonsentrasies is aangetoon. Hippokampale NA, DA en 5-HT was verhoog op dag 7 na TS ($p < 0.05$). Op dag 7 na HS, was hippokampale 5-HT statisties beduidend laer ($p < 0.05$). Alhoewel geen konstante MA veranderinge tydens hierdie studie aangetoon is nie, is dit duidelik dat die TAS model monoamien disregulasie veroorsaak. Gedragsanalise dui daarop dat die rotte op hulle angstigste was op dag 7 na HS.

Omdat stress-verwante angs nie behoue gebly het na die TS nie, maar wel na die HS ($p < 0.05$), dui die resultate daarop dat die TAS model wel PTAS-agtige simptome in rotte induseer en dat die HS nodig is vir die behoud van die stres respons. Hierdie model blyk dus nuttig te wees in die verdere soektog na potensiële geneesmiddels vir die behandeling van PTAS.

Sleutelwoorde: Posttraumatiese angs steurnis (PAS), kortikosteroon, tyd-afhanklike sensitisasie (TAS) model, "elevated plus-maze (EPM)", hippokampus, pre-frontale korteks (PFK), monoamiene, rotte.

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I begin in the name of Allah, the Most Compassionate, the Most Merciful.

All Praise and Thanks are due only to Allah, Lord of All the Worlds. May Peace and Salutations be upon his Final Messenger Muhammad (SAW).

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<i>Congress Proceedings</i>	
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First Place Winner- Young Scientist Competition 2004.

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Ratio time in open arms = $100 \times \text{time in open arms} / \text{total time in arms}$ **(equation 5.3).**

Surface area ratio = $\text{AUC standard} / \text{AUC internal standard}$ **(equation 5.4)**

% RSD = $\text{Standard error variation (STDEV)} / \text{Mean AUC ratio}$ **(equation 5.5)**

List of abbreviations

α : alpha adrenergic receptors

β : beta adrenergic receptors

ACTH: Adrenocorticotropin hormone

ADH: Antidiuretic hormone

APA: American Psychiatric Association

cAMP: cyclic AMP

CBG: Corticotropin binding globulin

CNS: Central nervous system

CRF/CRH: Corticotropin-releasing hormone/factor

COMT: Catechol-O-methyl transferase

CORT: corticosterone

D₁₋₅: Dopamine receptor subtypes

DA: Dopamine

DAG: Diacylglycerol

DA- β -Hydroxylase: Dopamine beta hydroxylase

DOPAC: 3,4-dihydroxyphenylacetic acid

DSM-IV: Diagnostic Statistical Manual (fourth edition)

EPM: Elevated Plus Maze

5-HIAA: 5- Hydroxy-indole-acetic acid

5-HT: Serotonin

HPA: Hypothalamic-pituitary-adrenal axis

HSP: Heat shock protein

HVA: Homovanillic acid

11 β -HSD: 11 β -Hydroxysteroid dehydrogenase

GRs: Glucocorticoid receptors

GREs: Glucocorticoid responsive elements

IP₃:Inositol triphosphate

LC: Locus coeruleus

LDL PATHWAY: Low density liposacharide pathway

LTP: Long term potentiation

NA: Noradrenaline

NTS: Nucleus tractus solitarius

MA: Monoamines

MAO: Monoamine Oxidase

MAOIs: Monoamine Oxidase Inhibitors

M-C: Meso-cortical system

M-L: Meso-limbic system

MHPG: 3-metoxy-4-hydroxyphenylethylene glycol

MRs: Mineralocorticoid receptors

PFC: Pre-frontal cortex

PTSD: Posttraumatic stress disorder

PVN: Paraventricular nucleus

RS: Re-stress (situational reminder- swim stress)

SSRIs: Serotonin selective re-uptake inhibitors

TCAs: Tricyclic antidepressants

TDS: Time dependent sensitization Animal Model of PTSD

TS: Triple stressor (Restraint, swim and anesthetic stress)

VMA: Vanillylmandelic Acid

Introduction

Chapter 1

1.1 Problem Statement

Abnormal stressors are by no means a product of the modern era. They have always been a feature of human society. The psychological response to trauma is probably as old as human nature, however the role of coping following a distressing event has received considerable research attention over recent years.

Although most often, experiencing a traumatic event does not lead to long term psychological problems, some individuals continue to experience trauma related symptoms thereafter, often qualifying for the diagnosis of posttraumatic stress disorder (PTSD).

Formalised as a disorder only in 1980, posttraumatic stress disorder, according to the Diagnostic and Statistical Manual of Psychiatric Disorders (DSM-IV), is a pathological anxiety disorder characterized by the **re-experiencing** of the traumatic event, **hyper-arousal** and **avoidance** of the stimuli associated with the trauma (American Psychiatric Association, 1994).

Patients diagnosed with PTSD form a specific association between the stress of the trauma and alteration in memory function (Pitman, 1989) thus exhibiting persistent involuntary reliving of bygone events. This anxiety disorder is thus primarily a disorder of memory (McNally, 1998).

Anxiety occurs when the information perceived by the individual and those already in memory mismatch (Beuzen & Belzung, 1995). The emotional disturbances of PTSD have been suggested to have their origin in the inability of the prefrontal cortex (PFC) and the hippocampus to modulate amygdala function (Gore & Richards, 2002).

Many of the hallmark symptoms of PTSD for example nightmares, flashbacks, and exaggerated startle response represent at least in part, disturbances in neurocognitive processing. In particular sensory input and memory processing seem awry (Newport & Nemeroff, 2000).

The medial prefrontal cortex is that particular area of the brain that plays an important role in the process of fear conditioning, specifically with regard to the termination of the fear response

(LeDoux, 1998). Damage to, or hypofunction of this area produces prolonged extinction, resulting in fears that are difficult to get rid of once established.

The medial prefrontal cortex is also the target site for the negative-feedback effects of glucocorticoids during the stress-induced activation of the hypothalamic-pituitary-adrenal (HPA)-axis suggesting its importance in the stress response (Diorio *et al.*, 1993). HPA-axis abnormality suggests dysregulation in prefrontal cortical activity (Sullivan & Gratton, 2002).

Although the hippocampus regulates corticosterone release through its inhibitory actions on the HPA-axis (Bremner, 1999), hippocampal damage would result in the disruption of the negative feedback loop, thereby making the hippocampus increasingly vulnerable to the toxic effects of glucocorticoids (Nutt, 2000). The decreased hippocampal volumes observed in patients with PTSD have been in part ascribed to increased exposure of the hippocampus to glucocorticoids (Sapolsky, 2000).

PTSD is a disorder associated with an enhanced negative feedback mechanism of cortisol on the HPA-axis (Liberzon *et al.*, 1997). Some, but not all, studies have reported a peripheral hypocortisolism (Harvey *et al.*, 2003). Other findings do not support the concept of either a static "hypo/hyper-cortisolism" in PTSD but rather suggest a psychogenic basis for cortisol alterations in relation to psychosocial stress. This is indicative of a central regulatory dysfunction of the hypothalamic-pituitary-adrenal axis, characterized by a dynamic tendency to overreact in both upward and downward directions (Mason *et al.*, 2002).

Studies have established a regulatory role of serotonin in HPA-axis function, with the 5-HT_{1A} receptor being implicated in particular. Qualitative and quantitative increases in hippocampal 5HT_{1A} receptors may account for the disturbance in memory and emotion (Harvey *et al.*, 2003).

Alteration in the metabolism and secretion of the monoamines noradrenaline and dopamine has also been reported in PTSD patients (Charney *et al.*, 1993). Patients diagnosed with PTSD, excrete significantly greater amounts of urinary dopamine that is correlated most directly with intrusive symptoms of PTSD (Freeman *et al.*, 2002). Evidence of dysregulation of the noradrenergic system is confirmed by the exaggerated increase in heart rate and blood pressure when patients diagnosed with PTSD are exposed to visual or auditory reminders of the trauma (Southwick *et al.*, 1999).

Although PTSD is a condition that presents in humans, animal models are useful in the study of the response to severe stress. The Time Dependent Sensitisation (TDS) model, based on the principle of exposure to three intense consecutive stressors (TS) followed by a mild situational

reminder (RS) seven days thereafter, is a valid rodent model of stress. Previous findings have indicated that this rodent model displayed the most profound PTSD-like symptoms in Sprague-Dawley rats 7 days after being exposed to the situational reminder (RS) (Naciti, 2002).

The aim of this study was to further validate the TDS model and to characterize the development and progression of PTSD using anxiety-like behaviour, plasma corticosterone concentrations and monoamine (dopamine, noradrenaline and serotonin) concentrations in the hippocampus and pre-frontal cortex (PFC).

1.2 Study aim and lay-out

The TDS model uses three acute stressors (TS) of intense severity followed by a mild situational reminder (RS) on day 7 subsequent to the acute stressors. The rationale that prior exposure to a trauma (TS) is an important risk factor for the development of PTSD and consequent exposure to a mild situational reminder (RS) causes the maintenance of the fear response over time (Uys *et al.*, 2003).

The aims of this study were to:

Determine the difference in stress response to the TS and the RS to further validate the TDS model at various points during and after the stressors in order to characterize the development and progression of stress-related pathology. Methods used to achieve these aims were:

- High performance liquid chromatography (HPLC) with electrochemical detection to quantify monoamine neurotransmitters (noradrenaline, dopamine, serotonin and metabolites) concentrations in the pre-frontal cortex and the hippocampus (biochemical correlates);
- Radio immuno assay (RIA) to determine plasma corticosterone concentrations (neuroendocrine parameter);
- The usage of the Elevated Plus Maze (EPM) to confirm the absence or presence of anxiety-like behavior (behavioral analyses).

The current study examined the changes that could have occurred at time intervals prior to and including day 7 post RS. Day 7 post situational reminder had been identified as the day on which the most profound PTSD-like symptoms were noted in a previous study (Naciti, 2002).

This project lay-out consisted of two studies:

1. In the **Acute Study**, three groups of ten rats were exposed to the **Triple Stressor (TS)**. *Group 1* was sacrificed on the day of TS exposure (TS stat); *Group 2* sacrificed 3 days after TS and *Group 3* sacrificed 7 days post TS.
2. The **Re-Stress Study**, in which rats were exposed to the **Triple Stressor (TS)**, left undisturbed for 6 days and **then exposed to a situational reminder (RS) on day 7**. This study also had three groups of ten rats each. *Group 4*, was sacrificed on the day of RS exposure (RS stat), *Group 5* was sacrificed 3 days post RS, and *Group 6* was sacrificed 7 days post RS.

A group of ten rats were included in the study as unstressed **Control**.

In the current study clarity was sought to determine as to the capability of the TDS model in inducing monoamine dysregulation. This would undoubtedly increase the model's utility in studying and understanding the pathological changes that underlie exposure to severe stress.

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Posttraumatic stress disorder (PTSD)

Chapter 2

2.1 Introduction

Posttraumatic stress disorder (PTSD) may result from exposure to an extremely traumatic life-event (Engelhard *et al.*, 2003). It is a disorder directly precipitated by an event that threatens the physical integrity or life of an individual or that of another individual (Gore & Richards, 2002).

Although individuals ascribe different levels of significance to a given event, all patients must have symptoms that are related to the trauma (Pine *et al.*, 1998). In PTSD there is an association between the extreme stress of the trauma and memory function alteration (Pitman, 1989). Posttraumatic stress disorder is thus primarily a disorder of memory (McNally, 1998).

The Diagnostic and Statistical Manual of Psychiatric Disorders (DSM-IV) classifies PTSD as an anxiety disorder, comprising of three symptom clusters:

- 1 **re-experiencing** the event,
- 2 with the resultant symptoms of numbness and **avoidance**, and
- 3 **hyper arousal** (American Psychiatric Association, 1994).

Patients diagnosed with PTSD **re-experience** the traumatic event by reliving the event in the form of nightmares, flashbacks and re-current daytime memories. They **avoid** reminders such as the place, memories, activity and thoughts associated with the specific event. **Hyper arousal** symptoms that may be present are insomnia, lack of concentration and irritability (Engelhard *et al.*, 2003).

Once a diagnosis shrouded in controversy, PTSD is now a valid diagnostic entity with a significant database of neurological research (Newport & Nemeroff, 2000).

2.2 Symptomatology of PTSD

The clinical presentation of PTSD is often very heterogeneous and symptoms differ in intensity from patient to patient (Albucher & Liberzon, 2002). Experiencing, witnessing or being confronted with an event involving serious injury, death or threat to the physical integrity of an

individual, along with a response of loss of control, helplessness, intense fear or horror may cause PTSD (Gore & Richards, 2002).

Symptoms of PTSD may be difficult to distinguish from panic or generalized anxiety disorder since all three syndromes are associated with prominent anxiety and autonomic arousal. The **characteristic symptoms of PTSD** associated with **re-experiencing and avoidance** of the trauma are not typically associated with panic or generalized anxiety disorder (Gore & Richards, 2002).

The DSM-IV, defines the essential features of PTSD as the development of the characteristic symptoms following exposure to an extreme traumatic stressor. The following criteria have been outlined (APA, 1994):

1. The first major criterion has two components:

- Experiencing, witnessing, or being confronted with an event that involves injury, death, or a threat to a person's existence.
- A response involving intense fear, horror or helplessness.

2. The second major criterion involves persistent re-experiencing of the trauma in the form of flashbacks, dreams, images, and hallucinations.

3. The third major diagnostic criterion involves the avoidance of stimuli that are associated with the trauma and numbing of general responsiveness. This is determined by the presence of three or more of the following:

- Avoidance of thoughts, feelings, or conversations that is associated with the event.
- Avoidance of people, places, or activities that may trigger recollections of the event.
- Inability to recall important aspects of the event.
- Significantly diminished interest or participation in important activities.
- Feeling detachment from others.
- Sense of foreshortened future.

4. The fourth criterion which is that of increased arousal requires two or more of the following symptoms:

- Difficulty in falling asleep or sleeping.
- Decreased concentration.

- Hyper vigilance.
- Outbursts of anger or irritable mood.
- Exaggerated startle response.

The duration of the relevant criteria should be more than one month and the disturbance should be a cause of significant distress or clinical impairment (American Psychiatric Association, 1994).

A PTSD diagnosis, according to Gore and Richards (2002) can be classified as one of the following:

- *Acute*: PTSD symptoms lasting less than 3 months.
- *Chronic*: symptoms of PTSD lasting 3 months or longer.
- *Delayed onset*: A period of 6 months between the traumatic event and the onset of symptoms.

2.3 Factors that may influence the severity of PTSD symptoms

Although a stressor is necessary, it is not sufficient to cause the disorder. The majority of people do not develop PTSD even when faced with overwhelming trauma. According to Kaplan and co-workers (1994), PTSD can develop in some people in response to mundane and less catastrophic events because of subjective meanings attached to that particular event.

In considering the diagnosis of PTSD, pre-existing biological and psychosocial factors as well as events subsequent to the trauma need to be considered. The below mentioned factors increase the probability of developing PTSD:

- **Pre-trauma vulnerability**

Pre-trauma vulnerability encompasses genetic and biological risk factors, as well as factors related to the life course of the individual, mental health and personality (Shalev, 1996). Personality traits such as neuroticism, introversion and prior mental disorders increase the risk of developing PTSD (Shalev, 1996).

♦ **Magnitude of the stressor**

The intensity of the traumatic event, expressed in terms of combat intensity and duration, dangerousness of a rape incident, intensity of torture experienced or extent of physical injury contributes significantly to the development of PTSD (Shalev *et al.*, 2001).

♦ **Post-trauma response**

During the days and weeks following the impact phase of the trauma, patients show a variety of effects that are associated with the development of PTSD. Distress is always present. According to Shalev (1996) symptoms resembling those of PTSD are frequently observed during the early days following the trauma. Intrusive re-collection of the event appears within 48 hours of the event in many of the survivors. Moreover PTSD patients report higher levels of intrusion, avoidance, depression and state anxiety in the week that followed the trauma (Shalev, 1996).

Not all people exposed to a extremely traumatic life event develop PTSD. However, PTSD can occur at any age, including childhood. Females with a history of sexual assault are at a higher risk of developing PTSD, while trauma from combat has the highest impact in men that develop PTSD (Gore & Richards, 2002).

Others more likely to develop PTSD include those (Kaplan *et al.*, 1994):

- With a border line, paranoid, dependent or antisocial personality disorder trait.
- Who report a greater perceived threat or danger.
- Those within a social structure that promotes shame, guilt, stigmatisation or self hatred.
- Those with prior vulnerability symptoms such as genetics, lack of functional and social support and concurrent stressful life events.

2.4 Neuroanatomy of PTSD

Many of the hallmark symptoms of PTSD for example nightmares, flashbacks, and exaggerated startle response represent at least in part, disturbances in neurocognitive processing. In particular sensory input and memory processing seem awry (Newport & Nemeroff, 2000).

Disturbances in sensory perception are believed to play a prominent role in the hyper arousal symptoms of PTSD such as the exaggerated startle response (Newport & Nemeroff, 2000). Reduced hippocampal volume causes behavioral disinhibition and it is likely that this accounts for the memory deficits seen in patients with PTSD (Bremner *et al.*, 1995).

Many new tools, such as neuropsychological testing, polysomnography and various modalities for functional brain imaging, including single proton emission computed tomography (SPECT), positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) are available for investigating neurocognitive processing in PTSD (Newport & Nemeroff, 2000).

It is likely that emotions evolved from simple mechanisms that gave animals the capacity to avoid harm. Consequently, understanding emotional processing in animals may offer insight into the neurobiology of human emotion (Cardinal *et al.*, 2002). Understanding the relationship between brain structure, function and the disturbances that occurs in mental illness is one of the major challenges that an experimental neuroscientist faces (Leonard, 1997).

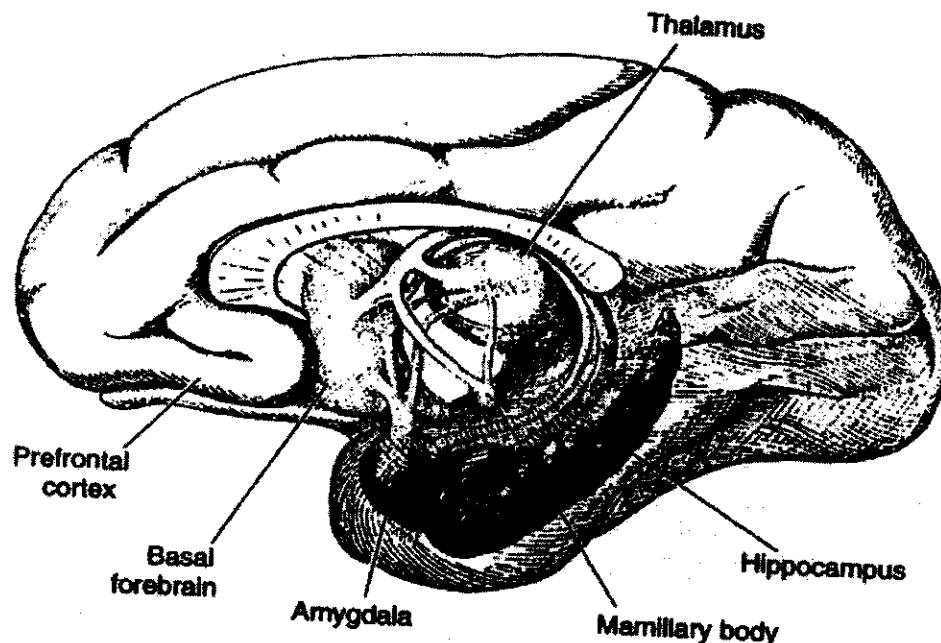


Figure 2.1: Main regions of the human brain concerned with memory, emotion and intellect (Leonard, 1997).

It is now becoming clearer that we are not looking for distinct anatomical sites but instead for the neural circuit that underlies PTSD. Multiple brain structures are involved in the organisation of response to aversive or stressful stimuli.

The limbic system is that part of the central nervous system reported to maintain and guide emotions and behaviour necessary for self-preservation and survival (Elzinga & Bremner, 2002). The emotional disturbances of PTSD have been suggested to have their origin in the

inability of the **prefrontal cortex (PFC)** and the **hippocampus** to modulate **amygdala** function (Gore & Richards, 2002).

2.4.1 The amygdala

2.4.1.1 Structure

The limbic system has a central role in the genesis of emotion. Within the limbic system lies the amygdala (Sandford *et al.*, 2000).

The amygdala is composed of several nuclei, which perform different functions. The lateral and the basolateral nuclei of the amygdala funnel and integrates sensory input from the thalamus and cognitive information from the cortex and hippocampus (van der Kar & Blair, 1999).

It has extensive communications as well as interconnections to the cortex and the locus coeruleus and projections to the striatum, midbrain and the brainstem. This means that the amygdala exerts control over locomotor, neuroendocrine, autonomic and respiratory responses (Sandford *et al.*, 2000).

2.4.1.2 The role of the amygdala in PTSD

The amygdala has been implicated in the expression, conditioning and extinction of fear. It plays a significant role in consolidating the emotional significance of events and is the key brain structure associated with PTSD (Gore & Richards, 2002). This region has a crucial role in emotional processing (Cardinal *et al.*, 2002) and in fear conditioning that is important in PTSD (Newport & Nemerhoff, 2000).

Noradrenergic activation within the amygdala seems to be critically involved in the regulation of processes modulating neural plasticity in other brain regions (Ferry *et al.*, 1999). The amygdala is responsible for modulating memory storage in both the hippocampal and caudate nucleus dependent tasks (Ferry *et al.*, 1999).

Damage to the amygdala in humans may lead to an increase in the threshold of emotional perception and expression. Increasingly the amygdala is being seen as the common pathway and processor of fear (Bremner, 1999).

2.4.2 The hippocampus

2.4.2.1 Structure of the hippocampus

The hippocampal formation is a portion of the cerebral hemisphere located above the corpus callosum and the thalamus. The position of the hippocampus relevant to other brain structures is illustrated in **Figure 2.1**. At cellular level the hippocampus consists of pyramidal neurons: CA₁, CA₂ and CA₃, which are not actual structures but rather designated anatomical areas (Molavi, 1997).

2.4.2.2 The role of the hippocampus in PTSD

The hippocampus plays an important role in new learning and memory (Zola-Morgan & Squire, 1990). Patients with PTSD demonstrate a variety of memory problems including deficits in recalling facts and fragmented memory (Bremner, 1999). They also may display trauma-related amnesia (gaps in memory that occurs for minutes to days that are not due to ordinary forgetting) (Elzinga & Bremner, 2002).

This hippocampus has the ability to regenerate nerve cells as part of its normal functioning, but stress impairs this function by stopping or slowing neuron regeneration (Elzinga & Bremner, 2002). Stress related hippocampal atrophy appears to be a consequence of increased exposure to glutamate, GABA and glucocorticoids (Newport & Nemerhoff, 2000).

Animal studies show direct glucocorticoid exposure results in decreased dendritic branching (Wooley *et al.*, 1990), alteration in synaptic terminal structure (Magaritanos *et al.*, 1997), a loss of neurons (Uno *et al.*, 1990) and an inhibition of neuronal regeneration (Gould *et al.*, 1998) within the CA3 region of the hippocampus.

Although the hippocampus regulates corticosterone release through its inhibitory actions on the HPA-axis (Bremner, 1999), hippocampal damage would result in the disruption of the negative feedback loop, thereby making the hippocampus increasingly vulnerable to the toxic effects of glucocorticoids (Nutt, 2000).

Right-sided hippocampal atrophy has been reported with adult PTSD patients with measurable deficits in verbal recall (Bremner *et al.*, 1995). The decreased hippocampal volume observed in patients with PTSD has been in part ascribed to increased exposure of the hippocampus to glucocorticoids (Sapolsky, 2000).

2.4.3 The Pre-frontal cortex (PFC)

2.4.3.1 Structure

The cerebral cortex is the most highly developed part of the human brain and is responsible for thinking, perceiving and understanding language. It can be divided into areas that have specific functions such as vision, hearing, touch and movement (Leonard, 1997).

2.4.3.2 The role of the PFC in PTSD

The frontal cortex in humans, primates and rats is a large functionally heterogeneous region, thought to be important for the orchestration of a number of separate cognitive processes including working memory components and suppression of previous behavioural strategies. This executive function is believed to facilitate the matching of an appropriate behavioural strategy to rapid changes in task requirements (Broad *et al.*, 2002).

The PFC plays a seminal role in the working memory component of explicit memory and may play a counter-regulatory role in the stress response through its inhibitory effect upon the amygdala (Newport & Nemerhoff, 2000). It is held responsible for the enhanced traumatic memories and deficits in working memories of PTSD patients (Elzinga & Bremner, 2002) and plays a prominent role in inhibiting memories that are irrelevant to the stimuli.

NA exerts potent actions in the PFC thereby influencing memory (Berridge & Waterhouse, 2003). Elevated NA concentrations are known to impair PFC function resulting in deeply engraved traumatic memories that are expressed as flashbacks, nightmares and intrusive recollections (Pitman, 1989).

Excessive PFC dopaminergic activity, has a negative effect on cognitive function, resulting in inappropriate selecting and processing of environmental stimuli. Optimal cognitive functioning thus depends on an optimal range of dopamine turnover (Pani *et al.*, 2000).

PTSD patients have decreased emotional inhibition, increased intrusions and deficits in attention/concentration, due to the failure of the PFC to inhibit irrelevant cognition (Elzinga & Bremner, 2002). The excessive alertness seen in PTSD may be associated with increased demands on brain areas involved in visual-spatial aspects of memory function (Bremner *et al.*, 1995). Based upon the above mentioned, it is hypothesised that increased activity in the prefrontal cortex (involved in memory), may underlie the symptoms of PTSD (Bremner *et al.*, 1995).

Dysfunction of medial PFC, a structure that normally inhibits the activation of the amygdala, may further enhance the effects of amygdala function, thereby increasing the emotional valence and

frequency of intrusive memories (Elzinga & Bremner, 2002). Together the PFC and the amygdala play a role in stress sensitisation, thereby accounting for the effects of the prior trauma in the development of PTSD after subsequent traumatisation (Elzinga & Bremner, 2002).

2.5 Neurobiology of PTSD

Exposure to hostile conditions (usually referred to as a stressor) results in a co-ordinated effort to increase the chance of survival (Carrasco & van der Kar, 2002). This co-ordinated effort (stress response) is composed of alterations in behaviour, autonomic function and secretion of multiple hormones including adrenocorticotrophic hormone (ACTH) and cortisol/corticosterone, adrenal catecholamines, oxytocin, prolactin and renin (van der Kar & Blair, 1999).

Noradrenaline neurons originating in the locus coeruleus (LC), as well as in other nuclei in the medulla and pons, are activated during stress and sends projections to cortical and subcortical regions. These neurons are believed to be important in mediating fear and fear responses (Hsiao & Potter, 1990). This system has been termed the LC-Noradrenaline system.

Projection sites of this system include the hypothalamus, septohippocampal system, amygdala, cingulate and the pre-frontal cortices (PFC), the nucleus tractus solitarius (NTS); A6 cell groups in the locus coeruleus, the parabrachial nucleus, cuneiform nucleus and the dorsal raphe nucleus (van der Kar & Blair, 1999).

The physiological changes associated with the stress response include:

Mobilisation of energy to maintain brain and muscle function, sharpened attention to perceived threat, increased glucose utilisation, enhanced cardiovascular output and respiration, immune function modulation, inhibition of sexual behaviour and decreased feeding (Sapolsky *et al.*, 2000). All of these responses are geared to increase the probability of survival.

The neuroendocrine response to stressors is considered as survival mechanisms during exposure to a life-threatening event (Carrasco & van der Kar, 2002). However chronic and persistent stress inhibits the stress response and induces desensitisation.

Chronic stress may permanently alter how an organism deals with its environment on a day-to-day basis (Yehuda *et al.*, 1991) Due to the nature of the stressor the HPA-axis may become tonically inhibited as adaptation to stress (Heuser & Lammers, 2003).

PTSD studies have focused primarily on two biological systems: the hypothalamic-pituitary-adrenal (HPA) axis and the catecholamine/sympathetic nervous system or the limbs of the locus coeruleus -noradrenaline/ autonomic system (Heuser & Lammers, 2003).

The actions of these two systems appear to be synergistic. Where the catecholamines facilitate the availability of energy to vital organs, glucocorticoids released from the adrenals act as the “anti-stress” hormone helping to shutdown or contains the neural responses initiated by the stress (Heuser & Lammers, 2003). **Adaptive responses** are the short activation of the HPA-axis, while **maladaptive responses** result in the over-production of stress hormones and a failure to terminate the activation of the HPA-axis (Heuser & Lammers, 2003).

Much still needs to be learned about the specific role of the different neurohormones in the stress response. The precise interaction between the HPA-axis and the catecholamines in the stress response is not entirely clear (**Figure 2.2**), glucocorticoids and catecholamines may modulate each others effects in stimulating active coping behaviour when release is simultaneous, while in the presence of lowered glucocorticoid levels undifferentiated fight/flight reactions may occur.

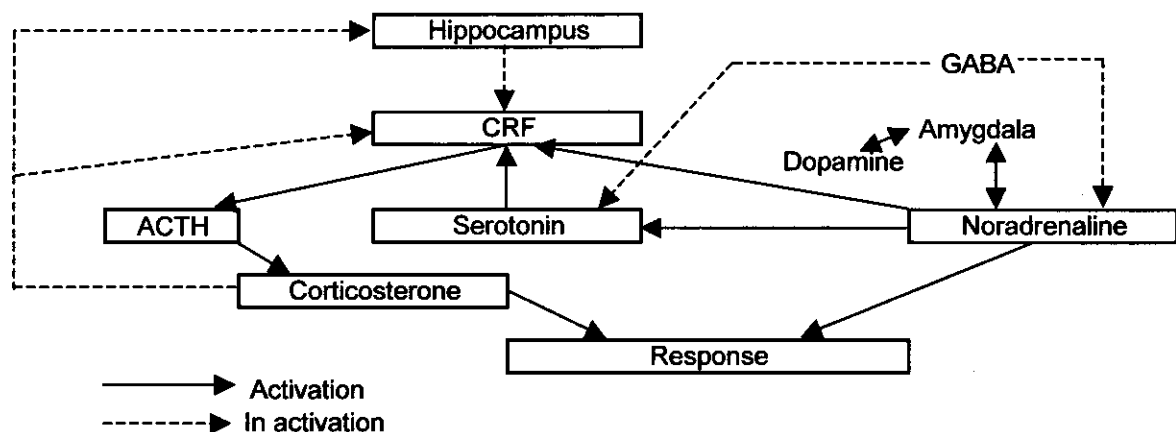


Figure 2.2: Brain circuits participating in the regulation of the neuroendocrine stress response. CRF=corticotrophin-releasing factor in the hypothalamic paraventricular nucleus; serotonin in the dorsal raphe nucleus; noradrenaline in the locus coeruleus; dopamine in the mesolimbic system; GABA=gamma-amino-butyric acid (Adapted from Carrasco & van de Kar, 2003).

HPA-axis alterations associated with PTSD include increased concentration of cerebrospinal fluid corticotropin -releasing factor (CRF), adrenocorticotrophic hormone (ACTH), low base line cortisol levels and alteration in the secretion of the monoamines (Heim *et al.*, 2001).

2.5.1 The hypothalamic-pituitary-adrenal (HPA)-axis

It is widely appreciated that the hypothalamic-pituitary-adrenal (HPA)-axis is of central importance to the individual in dealing with the stresses of life, be they social, physical or psychological (Sullivan & Gratton, 2002). Efficient activation and inhibition of the HPA-axis are essential components for optimal coping and long term well being.

The HPA-axis is activated following exhaustion, loss of control or the perception of loss of control (Chrousos & Gold, 1998). Stress induced activation of the HPA-axis involves a number of inter-related factors (De Souza & van Loon, 1982).

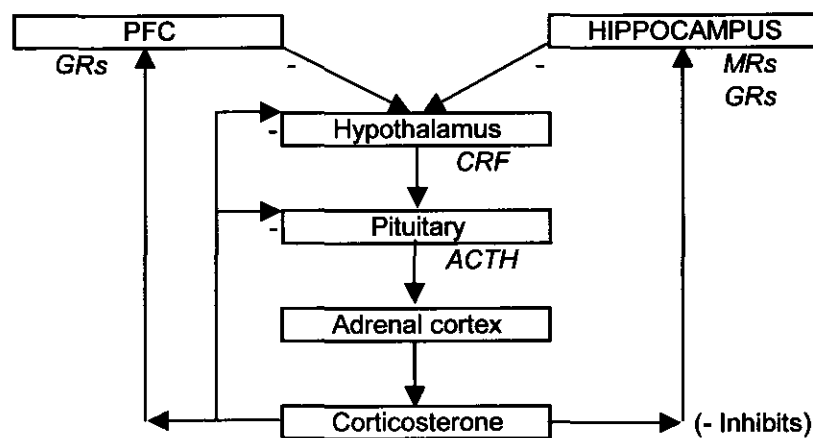


Figure 2.3: Schematic representation of the HPA axis (Adapted from Lupien & Lepage, 2001).

The physiological response to stress is largely mediated by an increase in the production and secretion of corticotropin releasing factor (CRF) /corticotropin releasing hormone (CRH).

CRF is a 41-amino acid polypeptide, which is derived from a 196-amino acid parent protein precursor is generated in numerous brain areas (Millan, 2003).

Of note, CRF neurons innervate noradrenergic centres in the locus coeruleus and the central nucleus of the amygdala. These areas are of recognised importance in anxiety and stress-responses (Carrasco & van de Kar, 2003).

CRF/CRH, which is released from the paraventricular nucleus (PVN) of the hypothalamus into portal circulation consequently, stimulates the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH). The primary neuroendocrine purpose of CRF is to increase the synthesis and release of ACTH (Lupien & Lepage, 2001).

The release of adrenocorticotrophic hormone (ACTH) from the pituitary results in the liberation of adrenal steroids (Koob, 1999). In PTSD, CRF is elevated, as demonstrated by high basal cerebrospinal fluid CRF concentrations obtained via a single lumbar puncture (Bremner *et al.*, 1997).

At the level of the brain, the activity of the HPA-axis is regulated by the hippocampus through the binary **Mineralocorticoid (MRs) and Glucocorticoid (GRs) Receptor systems** (refer to 3.6.3). There is considerable evidence for the importance of the limbic system, particularly the prefrontal cortex and the hippocampus exhibiting an inhibitory role on the HPA-axis (Feldman & Conforti, 1980). Both the PFC and the hippocampus contain corticosteroid receptors. The hippocampus contains both GRs and MRs while frontal lobes contain mostly GRs (Lupien & Lepage, 2001). Both cortical structures are able to inhibit the HPA-axis (**Figure 2.3**).

Along with inhibitory actions of the frontal and hippocampal region, circulating corticosteroids will act at the level of the hypothalamus to inhibit further secretion of CRF. A negative feedback mechanism exists between cortisol and the hypothalamus (Sapolsky *et al.*, 2000).

While the hypothalamic paraventricular nucleus (PVN) is considered the neural control center of HPA-axis activation and ultimately adrenal corticosteroid (*corticosterone in rats* is synonymous to *cortisol in humans*) release into circulation. Many extrahypothalamic structures are involved in either facilitating this activation or providing homeostatic feedback control (McEwen *et al.*, 1986).

HPA-axis abnormality suggests dysregulation in prefrontal cortical activity (Sullivan & Gratton, 2002). The consequence of these negative feedback loops will be a significant decrease of circulating levels of corticosteroids after the end of the stressor (Lupien & Lepage, 2001).

2.6 Pathophysiology of PTSD

Kolb (1987) was the first to propose that excessive stimulation of the Central Nervous System (CNS) at the time of the trauma may result in permanent changes in the brain and this may have a negative effect on learning, habituation and stimulus discrimination.

Patients with PTSD seem to suffer from a persistent activation of the biological stress response upon exposure to stimuli that remind them of their previously experienced trauma.

Based on animal models it has been widely assumed that massive secretion of neurohormones at the time of the trauma plays a role in the long-term potentiation (LTP) of traumatic memories (van der Kolk & van der Hart, 1991).

Allostasis refers to the process whereby stability is maintained through change via adaptive processes. By controlling all mechanisms simultaneously the brain can enforce command and determine physiological requirements. Allostatic load refers to the price the body may have to pay for being forced to adapt to an adverse physiological or psychological stressor that is present over time (Korte & De Boer, 2003). Chronic anticipation to a negative event with the involvement of corticosteroids may lead to allostatic load that has an important role in the development of anxiety disorders.

The acoustic startle response that consists of a characteristic sequence of muscular and autonomic response is mediated at both spinal and supraspinal levels (Davis, 1986) by excitatory amino acids such as glutamate and aspartate. An abnormal startle response reflects an inability of PTSD patients to properly integrate memories of trauma (van der Kolk, 1994).

2.7 Psychophysiology of PTSD

In considering the relationship between trauma exposure and PTSD it is useful to distinguish between the acute and chronic response to a traumatic event. Most people who are exposed to a traumatic event develop symptoms in the early aftermath of the event (Yehuda *et al.*, 1998).

One of the most salient predictors of chronic PTSD is the nature of the traumatic event that has been experienced. Events associated with torture and prolonged victimisation is associated with the highest estimates for chronic PTSD (Yehuda *et al.*, 1998). At first glance it may be concluded that the development of PTSD is influenced by the adaptations occurring following the trauma. The ability to adapt and cope is a not psychological variable, but rather a biological variable (Yehuda *et al.*, 1998).

Abnormal psycho physiological responses in PTSD have been demonstrated on different levels i.e. in response to the specific reminders of the trauma and in response to intense but neutral stimuli (van der Kolk, 1994).

The first paradigm refers to heightened physiological arousal to sounds, images and thoughts related to the specific traumatic event. A large number of studies indicate that traumatised patients respond to such stimuli with significant conditioned autonomic reactions, such as increased heart rate, skin conductance and blood pressure (van der Kolk, 1994).

The highly elevated physiological responses that accompany the recall of the traumatic event illustrate the intensity and timelessness that accompany the response that affect current experiences (van der Kolk & van der Hart, 1991). Anxiety is a core symptom of PTSD.

2.7.1 Anxiety in PTSD

Anxiety occurs when the information perceived by the individual and those already in memory mismatch (Beuzen & Belzung, 1995). It is a normal emotion when appropriate to the environmental situation.

Symptoms of anxiety differ from individual to individual and could include headaches, palpitations and mild stomach discomfort. Anxiety has evolved biologically as a functional state and therefore can be both normal and pathological (Sandford *et al.*, 2000).

Patients with PTSD present with two kinds of anxiety symptoms (Cohen *et al.*, 2000). The first is **stimulus related** in which the patient experiences extreme distress upon exposure to a stimulus that provokes remembrance of the event but otherwise would avoid the feared object or situation. Flashbacks, where the individual feels and acts as if the trauma is recurring are the classic form of re-experiencing (Pine *et al.*, 1998). The second kind of anxiety symptoms includes worry, disturbed sleep and hyper-vigilance. These are **not stimulus related**.

Individuals often exhibit complex biological and behavioral responses to extreme trauma. Traumatized patients often develop mood disorders including major depression and other anxiety and substance abuse disorders (Pine *et al.*, 1998).

2.7.2 Memory in PTSD

Patients diagnosed with PTSD display a variety of memory problems. The APA (1994) identified two typical memory disturbances in traumatised patients: intrusive recollections and impoverished memory functioning.

Intrusive recollections are experienced as re-enactments of the original trauma and are accompanied by high levels of arousal; these memories are automatically triggered by situations that reflect aspects of the traumatic event (Elzinga & Bremner, 2002).

A "flashbulb" memory is the term used when reference is made to an intensely vivid memory related to an emotional event (Nutt, 2000). Dysfunction of the glutamatergic and GABA-nergic pathways have both been implicated particularly in the "flashbulb" memories of PTSD (Harvey, 1997).

Glutamatergic and GABA-nergic pathways in the brain are involved in the normal encoding of memory. This lead to the hypothesis that in PTSD there is an over stimulation of the NMDA receptor (Nutt, 2000). The NMDA receptor is an ion channel linked receptor of glutamate

(Cooper *et al.*, 1996). Over stimulation of the NMDA receptors will lead to the excessive influx of calcium ions, which eventually induce cell death in the hippocampus (Nutt, 2000).

Glutamate always has an excitatory input and GABA has an inhibitory input in the brain. A fine co-ordination between these two systems exists. The consequence of extreme stress is most likely mediated by the down regulation of the GABA-system, allowing an excessive activation of the glutamate system thereby facilitating long-term potentiation (LTP) (Nutt, 2000).

Glutamate transmission is involved in the registration of a threat, behaviourally responding to the threat and the physiological process (LTP) that is associated with learning. Both GABA and glutamate are believed to influence emotional states. GABA has been implicated in playing a role in anxiety and is an important part of the stress response (Nutt, 2000). GABA more importantly plays a role in the prevention of excessive NMDA receptor stimulation through the inhibition of glutamate transmission (Nutt, 2000).

It can be concluded that GABA and glutamate play an important role in the memory deficits that are profound in PTSD. Amnesia can be produced in animals that have altered glutamate activity suggesting a possible explanation for the memory deficits in PTSD (Glue *et al.*, 1993).

Patients with PTSD have impoverished declarative memory (Elzinga & Bremner, 2002). Declarative memory refers to the ability to consciously remember and reproduce events and facts. These patients exhibit memory deficits regarding sensory features of the trauma, emotional and physiological reactions experienced and the perceived meaning of the event (Elzinga & Bremner, 2002).

2.7.3 Brain Derived Neurotrophic Factor (BDNF)

BDNF belongs to the neurotrophic factor family that can be induced in response to neural activity. It has been shown to play a critical role in cellular models of learning and memory, particularly in long term potentiation (LTP) (Duman *et al.*, 1999).

Neurotrophic factors act on receptors referred to as Trks that contain an extracellular binding domain and an intracellular tyrosine kinase domain. Binding of two molecules of BDNF to TrkB results in dimerisation and activation of the intracellular kinase domain resulting in the phosphorylation of the receptor (Duman *et al.*, 1999) causing the activation of the MAP kinase pathway that is independent of second messenger systems or via the cAMP and calcium ion dependent pathway.

Expression of BDNF in the hippocampus has been found decreased in response to immobilisation stress in animals (Smith *et al.*, 1995). The mechanism underlying the influence of

stress on BDNF expression has not been fully characterised. It has been suggested that down regulation of BDNF can be attributed to elevated corticosterone levels (Duman *et al.*, 1999). Smith and co-workers (1995) reject the hypothesis that elevated corticosterone levels are responsible for the decreased expression of BDNF, due to the finding that hippocampal BDNF expression is found reduced in response to stress even in the absence of the adrenal gland.

Another possibility is that monoamine systems, such as noradrenaline and serotonin are activated by stress and this contributes to decreased BDNF expression. Vaidya and co-workers (1999) have reported a down regulation of BDNF following the administration of a serotonin antagonist. This could possibly be explained by the activation of GABA-ergic interneurons that inhibit neuronal activity in the hippocampus. A shift in the GABA/glutamate balance leaning more towards GABA, could favour the decreased expression of BDNF in the hippocampus (Smith *et al.*, 1995).

It has been suggested that BDNF could influence the monoamine system actions via increasing the function of the monoamine neurons or increased output of target neurons (Duman *et al.*, 1999).

2.8 Other Neurotransmitter systems involved in PTSD

Corticosterone released during emotionally arousing events activate noradrenergic mechanisms resulting in enhanced memory of that event (Ferry *et al.*, 1999). Increased levels of cortisol bring about an increase in CNS serotonin turnover by increasing tryptophan availability and stimulating tryptophan hydroxylase (Davis *et al.*, 1995). There is evidence that biological dysregulation of the serotonergic (5-HT), noradrenergic (NA) and dopaminergic (DA) central pathways play a central role in the stress-related pathology of PTSD. These changes will be discussed in detail in chapter 3.

The noradrenergic system contains centrally acting neurons that play a critical role in alertness, vigilance, memory and fear conditioning to life threatening stimuli (Southwick *et al.*, 1999). Evidence of dysregulation of this system is confirmed by the exaggerated increases in heart rate and blood pressure when patients diagnosed with PTSD are exposed to visual or auditory reminders of the trauma (Southwick *et al.*, 1999).

Heightened dopaminergic activity seems to be present in PTSD (Freeman *et al.*, 2002). With PTSD, patients excrete significantly greater amounts of urinary dopamine which may be correlated to the intrusive symptoms of PTSD (Freeman *et al.*, 2002). Although involvement of the dopamine system in anxiety disorders may be complex, preliminary evidence supports excessive dopaminergic activity in symptomatic states of mania and anxiety (Freeman *et al.*,

2002). Fearfulness is a key symptom in PTSD and may trigger the intrusive recollections that in turn enhance anxiety

The serotonergic system appears to be involved in the cognitive impairments noticed in PTSD (van Praag, 2004). The diversity of the symptoms of PTSD suggests the involvement of multiple neurobiological systems. In the past twenty years psychology and neuroscience have begun to elucidate the intricate interplay between the underlying biological substrate of the individual and the complex human experience of trauma (Albucher & Liberzon, 2002).

The inter-play between the different roles of the monoamines and corticosterone in PTSD are complex and merit further discussion (see chapter 3). Despite this complexity advances in neurobiology have shed some light on the neurobiological systems that may be altered by severe stress (Albucher & Liberzon, 2002), in turn this has made the pharmacological intervention in changing the course of the disorder possible.

2.9 Treatment of PTSD

Initiating the assessment and treatment quickly after the traumatic event may prevent many complications and disabilities associated with PTSD.

Therapy is often complicated by the presence of co-morbid disorders such as alcohol or substance abuse that should be treated first. However in the presence of major depression treatment should focus on PTSD since the course, biology and treatment response are unlike that of major depression (Gore & Richards, 2002). Treatment is best accomplished by a combination of both psychotherapy and pharmacotherapy.

Psychotherapy consists of group therapy, individual and family therapy, anxiety management and relaxation techniques. Hypnosis has also been used successfully (Gore & Richards, 2002). Given that severe stress influences many system within the brain, various drug classes have successfully been used in the treatment of PTSD.

2.9.1 Benzodiazepines

It is hypothesised that early treatment of trauma victims with benzodiazepines may offer protection towards future development of PTSD. These drugs have a rapid onset of anti-anxiety effect (Albucher & Liberzon, 2002).

Open trials with the benzodiazepines clonazepam and alprazolam have been conducted but patients presented with severe withdrawal symptoms (Friedman, 1998) and increased risk of dependence. According to Argyropoulos and co-workers (2000) benzodiazepines are ineffective in treating the numbing and avoidance symptoms of PTSD.

2.9.2 Tricyclic antidepressants (TCA)

This class of drugs targets multiple neurotransmitter systems, thus TCA efficacy may be mediated by blocking the reuptake of serotonin and noradrenaline (Albucher & Liberzon, 2002). The TCAs amitriptyline and imipramine produced clinically significant improvement in the treatment of PTSD (Argyropoulos *et al.*, 2000).

2.9.3 Monoamine oxidase inhibitors (MAOI)

MAOIs inhibit the enzyme monoamine oxidase (MAO) and the efficacy of these drugs have been linked to the accumulation of serotonin, dopamine and noradrenaline (Albucher & Liberzon, 2002). Phenelzine, a MAOI, showed good improvement on the intrusive phenomena of PTSD, but the effect on avoidance symptoms was smaller. According to Friedman (1998) MAOI appear to produce moderate to good clinical improvement, primarily affecting the intrusive recollections, nightmares, and flashbacks. On the contrary, hyper arousal, numbing and avoidance behaviour are not affected. Clinicians are reluctant to prescribe these drugs for PTSD, given the high prevalence of alcohol and drug abuse in this category of patients (Argyropoulos *et al.*, 2000).

2.9.4 Selective serotonin-reuptake inhibitors (SSRIs)

Fluoxetine, a SSRI, has been studied extensively in the treatment of PTSD and shown the biggest effect when compared with other treatment outcomes (Albucher & Liberzon, 2001). It is useful in alleviating the core symptoms of PTSD such as hyper arousal, numbing, avoidance and intrusive re-experiencing of the trauma. Of particular interest is the ability of SSRI's to treat numbness, a symptom resistant to treatment so far (Friedman, 1998). The efficacy of this drug class stems from the enhancement of serotonergic function and subsequent modulation of anxiety (Albucher & Liberzon, 2001).

2.9.5 Buspirone

This anxiolytic is a partial serotonin agonist that decreases the function of postsynaptic 5-HT₂ receptors (Albucher & Liberzon, 2001). Its effects are noted by the ability to decrease anxiety, insomnia, flashbacks and depressed mood. The mean time to improvement was two weeks (Wells *et al.*, 1991).

2.9.6 Antipsychotics

According to Friedman (1998), the recent advent of atypical antipsychotics such as risperidone, clozapine and olanzapine may be of value in treating the psychotic symptoms of PTSD.

2.9.7 Mood stabilisers

The anticonvulsant drug, carbamazepine improved hostility, sleep impairment and poor impulse control in PTSD sufferers (Lipper *et al.*, 1986). The hyper arousal and hyperactivity symptoms improved significantly when treated with valproate (Fesler, 1991). Lithium, an element with many interactions within the CNS reduces irritability, anxiety, insomnia and nightmares (Kitchner & Greenside, 1985).

2.9.8 Adrenergic agents

Clonidine an adrenergic agonist, modulates adrenergic tone and produces improvement in arousal and intrusive recollections (Albucher & Liberzon, 2002). Used in combination with imipramine, improvement in sleep, startle response and nightmares have been reported (Argyropoulos *et al.*, 2000). Due to the possibility of developing tolerance, guafacine, another α_2 agonist with a longer half-life may be a useful alternative (Friedman, 1998).

Kolb (1987) reported that propranolol, a beta-blocker improved sleep, hyper arousal and startle responses, self-confidence, and psychological functioning. There was also a reduction in nightmares and other intrusive phenomena. This is surprising given the postulated adrenergic dysfunction in PTSD (Friedman, 1998).

2.10 Synopsis

PTSD is regarded as a chronic disorder, therefore by clinical consensus it is recommended that treatment be extended to between 6 months and a year following recovery (Argyropoulos *et al.*, 2000). Generally accepted factors predicting poor outcome are the presence of personality disorders and co-morbidity with other affective illnesses.

Treatment resistant PTSD does undoubtedly exist. Better understanding of factors leading to treatment resistance will produce effective means and strategies in dealing with them. Argyropoulos and co-workers (2000) suggest a general strategy that entails the management of external stressors, assessment of therapy compliance and combination with other treatments.

The fact that a variety of different classes of drugs are effective in the same disorder suggests a complex aetiology involving many neurotransmitter systems or perhaps a common pathway in

which the different actions of these drugs converge. The following chapter serves to further clarify the involvement of multiple neurotransmitter systems.

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Monoamines and corticosterone in PTSD

Chapter 3

3.1 Introduction

It has been suggested that monoamine dysregulation in the CNS is involved in the pathogenesis, development and progression of PTSD. Increased autonomic activity in PTSD has been associated with an increased release of **noradrenaline** (South, 2003). The **dopaminergic** system may also demonstrate sensitisation. In preclinical paradigms it can be shown that different stimuli, pharmacological and environmental, are cross-sensitisers of dopaminergic forebrain pathways. Patients with PTSD sometimes demonstrate symptoms of hyper vigilance and even paranoia, that are likely to be mediated by the dopaminergic system (South, 2003).

A number of studies also suggest **serotonergic** involvement in PTSD. In studies of fear conditioning, for example, serotonin appears to play an important role (Hensman *et al.*, 1991). Furthermore, serotonin is involved in stress-induced corticosteroid release (Joseph & Kennell, 1983). Finally, clinical overlaps between PTSD and depression, anxiety, impulsivity and aggression also suggest that serotonergic agents deserve study in the treatment of PTSD (Davis *et al.*, 1997).

The idea that PTSD does not involve the classical or normal stress response, but rather involves abnormal neurobiological processes, is also consistent with clinical knowledge. PTSD is in many ways a unique syndrome. Although increased release of **cortisol** might be expected in PTSD patients, some studies in fact demonstrate hypocortisolemia (Yehuda *et al.*, 1998). Other findings do not support the concept of either a static "hypo/hyper-cortisolism" in PTSD, but rather suggest a psychogenic basis for cortisol alterations in PTSD in relation to psychosocial stress (Mason *et al.*, 2002).

This indicates a central regulatory dysfunction of the hypothalamic-pituitary-adrenal (HPA)-axis characterized by a dynamic tendency to overreact in both upward and downward directions (Mason *et al.*, 2002). Furthermore, neurobiological sensitisation is consistent with clinical understanding of the adverse effects of early life events on subsequent responses to trauma (South, 2003).

It may already be clinically useful to try and relate some of the neurobiological dysfunctions found in PTSD to the different symptom clusters found in this disorder (Charney *et al.*, 1993). Thus noradrenergic sensitisation presumably lies at the basis of hyperarousal symptoms. Dopamine dysfunction may mediate symptoms of hypervigilance and paranoia. Cortisol mediated damage to the hippocampus may underpin problems in memory. Such a scheme, although undoubtedly overly simplistic, may also lead to a heuristic approach to the pharmacotherapy of PTSD (Charney *et al.*, 1993).

3.2 Monoamines and their receptor binding mechanisms in the brain

Serotonin (5-HT), dopamine (DA) and noradrenaline (NA) are each made from a single amino acid, hence they are called "monoamine" (MA) neurotransmitters (South, 2003). Serotonin is built from the amino acid tryptophan, dopamine from phenylalanine and noradrenaline from tyrosine.

Monoamines as neurotransmitters share certain properties, but differ in their brain distribution, type of receptors they act upon and their mechanism of action. Noradrenaline (NA), adrenaline (Ad) and dopamine (DA) share a common synthesis pathway (Figure 3.1) with tyrosine acting as the precursor.

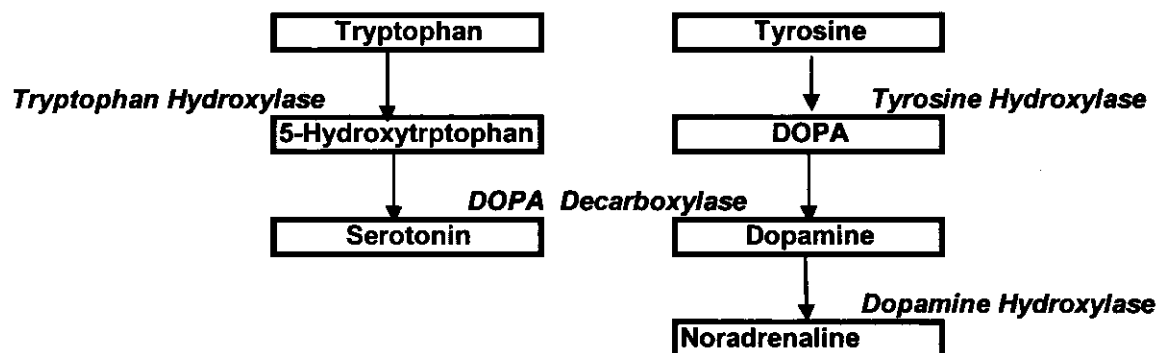


Figure 3.1: Synthesis of the noradrenaline, dopamine and serotonin (Adapted from Emson, 1983).

Phenylalanine is hydroxylated to tyrosine by phenylalanine hydroxylase (Rang *et al.*, 1999). Tyrosine is taken up from the blood stream and concentrated within the brain by active transport.

The activity of the enzyme *tyrosine hydroxylase* is regarded as the rate-limiting step in the synthesis of catecholamines because it regulates the formation of *L-Dopa* (Lefkowitz *et al.*, 1996). *L-Dopa* is then decarboxylated in the cytoplasm by the enzyme *L-Aromatic amino-acid decarboxylase* to form *dopamine (DA)* which in turn may be converted to NA by *dopamine-β-hydroxylase* (Bowen, 2003) (**Figure 3.1**).

The monoamine serotonin (5-HT) is synthesised by a two-step pathway from the essential amino acid tryptophan (**Figure 3.1**). Tryptophan hydroxylase, a mixed-function oxidase that requires molecular oxygen and a reduced pteridine cofactor for activity, is the rate-limiting enzyme in the pathway (Sanders-Bush & Meyer, 1996).

The enzyme aromatic L-amino acid decarboxylase converts *L-5-hydroxytryptophan* to *serotonin* (Sanders-Bush & Meyer, 1996) (**Figure 3.1**).

In order for a monoamine to exert an effect, receptor binding needs to occur. If the released monoamine interacts with a post-synaptic receptor that may result in a change in the membrane potential when receptors are coupled to ion channels. Such receptors are termed *ionotropic receptors*. If the receptor is coupled to a G-protein such receptors are known as *metabotropic receptors* (Elhwuegi, 2004).

If regulatory receptors are present on the same neuron as the neuron releasing the neurotransmitter, then it is called an *autoreceptor* and if the regulatory receptor is present on another neuron releasing a different neurotransmitter, it is referred to as a *heteroreceptor* (Elhwuegi, 2004). Regulatory receptors can be both ionotropic and metabotropic. Metabotropic receptors may be coupled to the cAMP transduction pathway.

The focus of this study revolves around the characterisation of the TDS model using the monoamine concentrations in the hippocampus and the PFC. The influence of serotonin, dopamine, noradrenaline and corticosterone in PTSD will be discussed in the pages that ensue.

3.3 Dopamine (DA)

Indeed, as compared to 5-HT and NA, rather little attention has been devoted to the role of DA in the response to, and in the modulation of anxious states (Stein *et al.*, 2002). In the late 1950's it was found that dopamine occurs in high concentrations in the central nervous system and that its distribution differs from that of noradrenaline (Carlsson, 1959). Dopamine was then investigated as an independent neurotransmitter and not only as a precursor of noradrenaline (Lavery, 1975).

3.3.1 Anatomical distribution of dopamine

Neurons containing dopamine are found in the nuclei of the hypothalamus and midbrain. Those in the arcuate nucleus project to the median eminence of the hypothalamus, forming the *tuberoinfundibular system*. Dopamine neurons in the substantia nigra project to the caudate nucleus and putamen (collectively referred to as the striatum) of the basal ganglia, forming the *nigrostriatal pathway*. Dopaminergic neurons in the ventral tegmental area project to the nucleus accumbens, amygdala, and prefrontal cortex to form the *mesolimbic (M-L)* and *mesocortical (M-C)* systems (Nicholls *et al.*, 1992). These are involved in adaptational processes (Smelik, 1987).

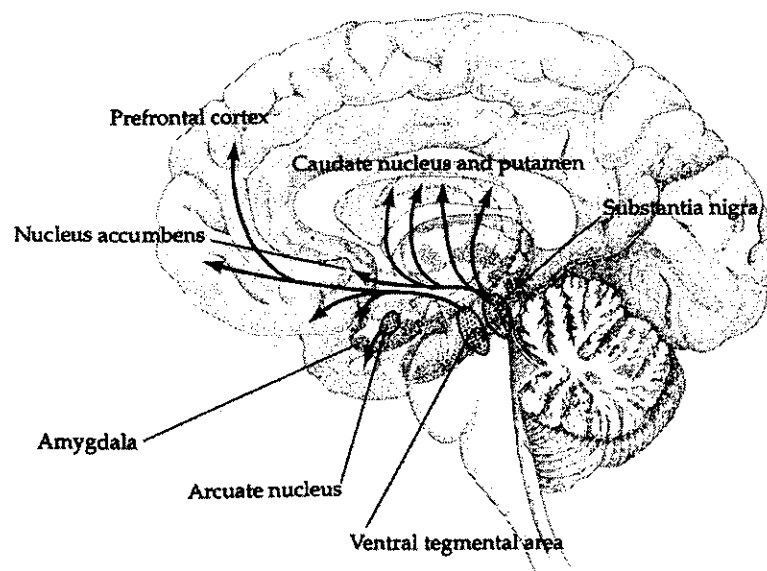


Figure 3.2: Distribution of dopamine in the brain (Nicholls *et al.*, 1992).

3.3.2 Dopaminergic receptors

Two types of receptors, D_1 and D_2 (linked respectively to the activation and inhibition of adenylate cyclase) were originally distinguished on pharmacological and biochemical grounds (Rang *et al.*, 1999).

The D_1 type includes the D_1 and D_5 . These receptors work on a signal transduction mechanism coupled to a G_s protein that increases cAMP production (Rang *et al.*, 1999) and its effect is mainly postsynaptic inhibition.

The D_2 type receptors include the receptors D_2 , D_3 and D_4 . These receptors decrease cAMP and/or increase IP_3 . Their effects include pre- and postsynaptic inhibition and the stimulation/inhibition of hormone release (Rang *et al.*, 1999).

The functional effects of dopamine D_2 receptor ligands in the brain arise from their interaction with dopamine D_2 expressed on dopamine neurons and with heteroreceptors on target cells (Elhwuegi, 2004).

3.3.3 Release and metabolism of dopamine

Dopamine that is released in the synapse interacts with both pre- and postsynaptic receptors. It may be actively **re-uptaken** in the presynaptic neuron, or it may **diffuse** out of the junctional cleft or it may under go **metabolic transmission**. (Lefkowitz *et al.*, 1996).

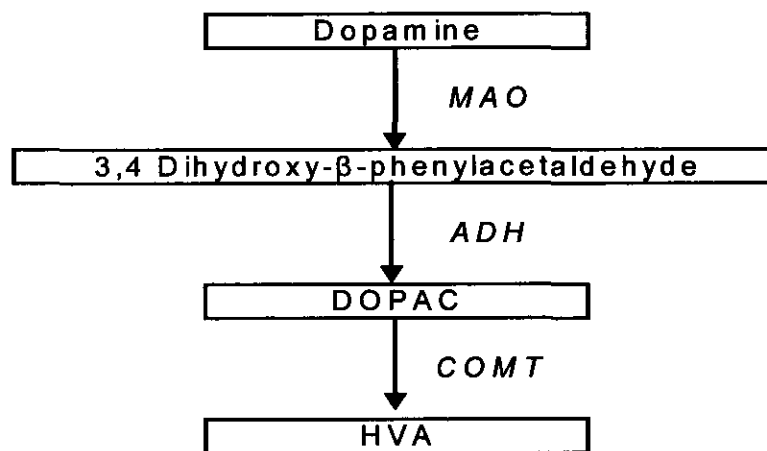


Figure 3.3: Inactivation of dopamine (Adapted from Nicholls *et al.*, 1992).

According to **Figure 3.3** two enzymes, monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), are important in the initial steps of metabolic transformation (Lefkowitz *et al.*, 1996). The main products that form are *dihydroxyphenylacetic acid* (DOPAC) and *homovanillic acid* (HVA) (Rang *et al.*, 1999).

3.3.5 The role of dopamine in PTSD

Dopaminergic pathways are important for reactivity to a disturbance in environmental conditions, selective information processing and general emotional responses all of which are necessary for the ability or failure to cope with the external world (Pani *et al.*, 2000).

These pathways fulfil an important role in the formation, retention and extinction of fear-related associations and memory (Morrow *et al.*, 1999). Although the precise neural circuit of dopamine

transmission remains unclear, pharmacological and neurochemical studies have pointed to the dopaminergic prefrontal neurons (Morrow *et al.*, 1999).

It seems that changes in dopaminergic transmission occur in reaction to particular threatening challenges (Reis *et al.*, 2004). The relationship of dopaminergic transmission to clinical anxiety disorders is complex and poorly understood. Empirical studies of dopamine involvement in patients diagnosed with PTSD have been inconclusive (Gelernter *et al.*, 1999) but dopamine has been implicated in the stress-related regulation of the HPA-axis (Millan, 2003).

Dopaminergic innervation of the prefrontal cortex is highly sensitive to stress (Deutch & Roth, 1990; Pani *et al.*, 2000). Acute and chronic stress both affect the central dopamine system. An increase in DA activity suppresses limbic DA transmission. Increased DA concentrations have been linked, most directly with intrusive symptoms of PTSD (Yehuda *et al.*, 1992).

However, excessive PFC dopamine activity has a negative effect on cognitive function, causing inappropriate selecting and processing of environmental stimuli. Optimal cognitive functioning thus depends on an optimal range of dopamine turnover (Pani *et al.*, 2000). Chronic stress may both reduce DA output (Imperato *et al.*, 1992) and increase dopamine turnover in the brain (Murphy *et al.*, 2003).

Indeed cortical dopamine projections are activated by several types of aversive stimulation. Behavioural studies aimed to disclose the involvement of dopamine in anxiety have reported anxiolytic-like, anxiogenic-like and lack of any effect with the use of dopaminergic agonists and antagonists in animal models of anxiety (Rodgers *et al.*, 1994).

An alteration in dopamine transmission was reported following exposure to a wide variety of acute stressors. Reis and co-workers (2004) found that both the D₁ and D₂ receptors are involved in the acquisition of conditioned avoidance response. Activation of the D₂ receptors occurs in the setting up of adaptive responses to innate fear stimuli, while a combined activation of D₁ and D₂ seem to be involved in a conditioned fear response (Reis *et al.*, 2004).

Dopamine systems are well known to play a role in the emotional response to rewarding as well as aversive stimulation (Le Moal & Simons, 1991). Decreased dopamine concentrations compromise the ability to develop effective coping strategies to dealing with trauma (Deutch & Young, 1995). Such a deficit may also contribute to hypervigilance and paranoia and greater susceptibility to trauma-related contextual stimuli (Gelernter *et al.*, 1999).

Rats exposed to uncontrollable stress have decreased dopamine levels as compared to unstressed rats for as long as two weeks after the removal of the stressor. These rats have a hypo-reactivity to both aversive and pleasurable stimuli (Lucas *et al.*, 2004).

In animal models, the administration of psychostimulants post-training has been reported to enhance memory, while drugs that decrease catecholamine levels such as reserpine, have been reported to produce memory impairment (McGaugh, 1989). A mixture of D₁ /D₂ agonist showed memory improvement when administered post-training. Further studies on the biochemical mechanisms involved and genetic analysis may provide more information on the role of dopamine in memory processes (Castellano, *et al.*, 1996).

Increased DOPAC concentrations indicate that MAO catabolised more DA, thereby increasing dopaminergic activity as a coping attempt to mild stress (Gamaro *et al.*, 2003). Sensitisation of the dopaminergic system of the PFC has been paralleled to kindling (4.3.2), and may be relevant to the pathogenesis of PTSD (Bonne *et al.*, 2003).

Injury to the nigrostriatal DA system results in few permanent behavioural changes unless the loss of tissue DA content in the striatum exceeds 95%. This phenomenon may be explained as an elevated rate of dopamine synthesis and release as well as a decreased re-uptake of DA. Stress induces a compensatory modification in DA neurons that permits continued responding (Abercrombie & Zigmond, 2000).

Yehuda and co-workers (1992) reported increased urinary dopamine excretion in patients diagnosed with PTSD as compared to healthy volunteers. Additional evidence of dopaminergic involvement in PTSD comes from a study showing an increased risk of developing the disorder associated with the D₂A₁ dopamine receptor allele (Comings *et al.*, 1996).

Severe stress does not suppress dopamine- β -hydroxylase activity, thus psychosis in PTSD might involve noradrenergic instead of dopaminergic hyperactivity (Hamner & Gold, 1998). Dopamine- β -hydroxylase activity may modify the phenomenology of PTSD by facilitating the experience of psychotic symptoms, but further work is needed to make a conclusive deduction (Hamner & Gold, 1998).

The monoamine noradrenaline, shares a common synthesis pathway with dopamine, but its effect and distribution in the body differs greatly.

3.4 Noradrenaline (NA)

Noradrenaline (NA) and adrenaline are monoamines that belong to a class of compounds known as the catecholamines. The term catecholamine refers to all organic compounds that contain a catechol nucleus and an amine ring (Cooper *et al.*, 1996). **NA** the main catecholamine in the postganglionic sympathetic nerves and in the CNS is released from the adrenal gland together with adrenaline (Leonard, 1997).

3.4.1 Anatomical distribution of noradrenaline

Figure 3.4 illustrates the distribution of noradrenaline in the human brain.

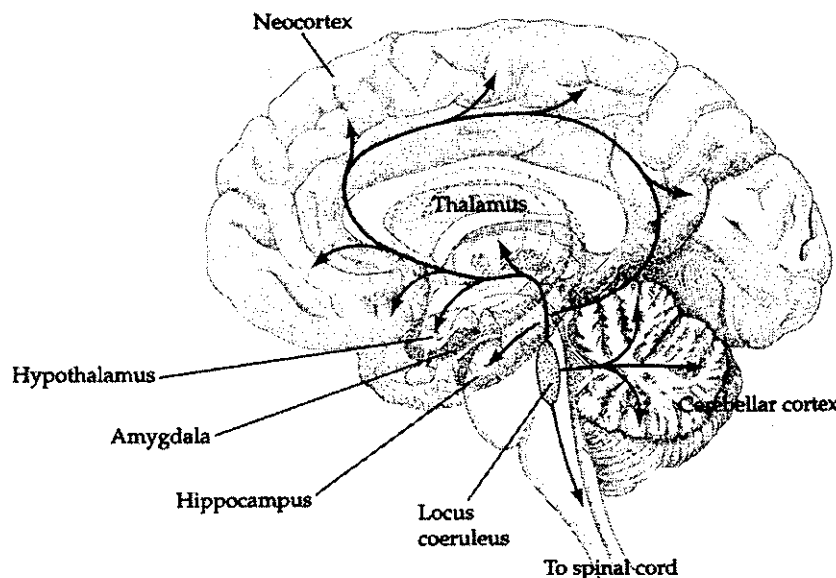


Figure 3.4. : Anatomical distribution of noradrenaline in the brain (Nicholls *et al.*, 1992).

Neurons containing NA project from the *locus coeruleus*. The locus coeruleus lies in the pons just beneath the floor of the fourth ventricle (Nicholls *et al.*, 1992). Noradrenergic projections ascend from the brain stem to innervate the thalamus, dorsal hypothalamus, hippocampus and cortex (Rang *et al.*, 1999).

3.4.2 Noradrenergic receptors

As early as 1948, designations α and β were proposed for receptors on smooth muscle where catecholamines produce excitatory and inhibitory responses respectively (Lefkowitz *et al.*, 1996). The α receptors are sub-divided as presynaptic α_2 and post-synaptic α_1 (Langer, 1997). The α_2 autoreceptor is coupled to the G_i -protein and inhibits adenylyl cyclase to decrease cAMP

formation (Rang *et al.*, 1999). The excitatory α receptors designated as α_1 activate phospholipase C, thus producing IP_3 and DAG as second messengers (Rang *et al.*, 1999).

The β receptors were later sub-divided as β_1 (those in myocardium) and β_2 (smooth muscle and most other sites) and β_3 (adipose tissue) receptors (Lefkowitz *et al.*, 1996). All types of β receptors are coupled to the G_s protein to stimulate adenylyl cyclase (Rang *et al.*, 1999).

3.4.3 Release and metabolism of noradrenaline

Once formed, NA is stored in highly specialised sub cellular vesicles in the CNS, sympathetic nerve endings and chromaffin cells (Rang *et al.*, 1999). These storage vesicles bind and store NA, slowing its diffusion out of the neuron, protecting NA from monoamine oxidase (Rang *et al.*, 1999).

Noradrenaline is released by exocytosis from nerve terminals during periods of nerve stimulation (Cooper *et al.*, 1996). Catecholamines are metabolised by two enzymes: catechol-O-methyltransferase (restricted to the synaptic cleft) and monoamine oxidase that occurs intraneurally (Rang *et al.*, 1999). The resultant compounds that are formed are: 3-Methoxy-4-hydroxy-phenylglycolaldehyde and 3,4-Dihydrox-phenylglycolaldehyde. These are further, metabolised to form 3-Methoxy-4-hydroxy-phenylglycol (MHPG) and 3-Methoxy-4-hydroxy-mandelic acid (VMA).

3.4.4 The role of the noradrenergic system in PTSD

The noradrenergic system acts as an arousal and alerting system, enhancing sensorimotor function in the response of an organism to external stimuli (Aston-Jones *et al.*, 1999). Activation of the brain noradrenergic system during stress response is thought to facilitate transmission in many brain regions facilitating specific behavioural and physiological processes (Jacobs *et al.*, 1991).

An inability to appropriately regulate or initiate the stress response has been proposed as a critical factor in the pathophysiology of PTSD (Sullivan, 1999).

Altered activity in noradrenergic systems has emerged as a key element of many aspects of pathological anxiety (Sullivan *et al.*, 1999). The NA-system interacts with the hypothalamic-pituitary-adrenal (HPA)-axis via CRF (Cullinan *et al.*, 1995). CRF derived from the paraventricular nucleus of the hypothalamus has a major role in the mediation of the stress response.

Evidence from an *in vivo* micro dialysis study indicates that NA stimulates CRF release under conditions of stress (Koob, 1999). As such, activation of the noradrenergic system plays an important integrative function in coping and adaptation to stress (Koob, 1999).

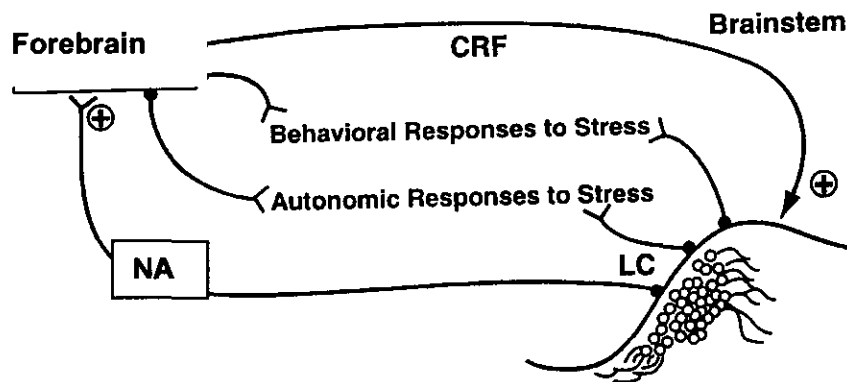


Figure 3.5: Schematic illustration of the interaction between CRF and NA (Koob, 1999).

The amygdala and its connections with the pre-frontal cortex play a key role in emotional memory. Increased noradrenergic release at the time of a traumatic event translates into enhanced consolidation and facilitated retrieval of emotional events (Carrasco, 1997).

Stress has been shown to produce regionally selective increases in noradrenaline turnover in the limbic region of the brain (Charney *et al.*, 2000). Extracellular NA concentration in the hippocampus measured under cold (3°C) resting conditions did not differ from rats under standard resting conditions (control). Exposure to a novel stressor caused an increased rate of NA release and synthesis in the experimental group as compared to control. From this it can be concluded that there are LC-NA neuron alterations in response to conditions of increased demands (Amercrombie & Zigmond, 2000).

NA-ergic hyperactivity may be associated with enhanced consolidation of emotion laden aversive events and memory disturbances seen in PTSD patients (van Praag, 2004). High levels of NA impair PFC function, resulting in deeply engraved traumatic memories that are expressed as flashbacks, nightmares and intrusive recollections (Pitman, 1989). Repeated re-

experiencing of the traumatic occurrences will enhance NA release, emotional reactivity and amygdala activity setting in motion a vicious cycle (van Praag, 2004).

It has been postulated that under conditions of stress, increased NA release stimulates the α_1 receptors that impair memory, but this is not necessarily detrimental to the animal, since increased NA levels are associated with higher levels of arousal that serve as important survival mechanisms (Berridge & Waterhouse, 2003). In response to excessive adrenergic function there may be post-synaptic down-regulation of adrenergic receptors (South, 2003).

Another study showed that acute stress results in increased tonic firing rates of NA neurons in the LC, decreased NA levels in the brain, increased NA turnover and increased extracellular NA levels (Abercrombie & Zigmond, 2000). The mechanisms by which both the HPA-axis and the LC-NA systems continue to function despite high corticosterone levels are unknown (Makino *et al.*, 2002).

Glucocorticoid receptors (GRs) are found in the LC, but the effect of corticosterone on catecholaminergic neural activity is still unclear. Corticosterone mediated inhibition of tyrosine hydroxylase (rate limiting enzyme in the synthesis of NA) in the LC and is likely to encourage NA release despite a robust hypercortisolemia (Makino *et al.*, 2002).

A body of evidence indicating the involvement of noradrenaline in memory processes comes from studies aimed at assessing the brain areas responsible for supplying noradrenaline-containing axons and terminals. Clinical reports have pointed to the critical role of the LC activation to improve memory loss in mice (Gold & Zornetzer, 1983).

Enhanced fear conditioning leads to fearfulness and hyper-arousal typical of PTSD (van Praag, 2004). After viewing of trauma-related videos blood pressure and heart rate rose in parallel with plasma NA and subjective distress (Yehuda *et al.*, 1998). Furthermore, patients show increased responsiveness to the α_2 autoreceptor antagonist, yohimbine. Considerable evidence indicates that noradrenergic function is abnormal in PTSD with elevated urinary excretion of catecholamines being reported in PTSD patients (Bonne *et al.*, 2003).

Increased NA levels are not sufficient to cause anxiety (Berridge & Waterhouse, 2003), but may be held responsible for the anxiety-like symptoms such as elevated blood pressure found in patients with PTSD.

Although chronic/repeated exposure to stressors do not necessarily result in increased NA release, they do result in an increased capacity of the system to release NA due to elevated rates of NA synthesis. CRF appears to participate in stressor induced up regulation of TH

synthesis in the LC under some circumstances. Chronic stress may result in increased biosynthesis of NA due to the apparent activation of tyrosine hydroxylase that persists following the termination of a stressful period (Abercrombie & Zigmond, 2000).

Repeated exposure to inescapable stress is associated with an eventual depletion of noradrenaline in the hypothalamus and hippocampus (Weiss et al., 1994).

3.5 Serotonin (5-HT)

Serotonin (5-HT) is a *neurotransmitter in the central nervous system (CNS)* and is also responsible for many different functions amongst others the control of mood; sleep patterns, temperature regulation, pain perception and the regulation of blood pressure (Sanders-Bush & Meyer, 1996).

3.5.1 Anatomical distribution of serotonin

Figure 3.6, illustrates the distribution of serotonin in the brain.

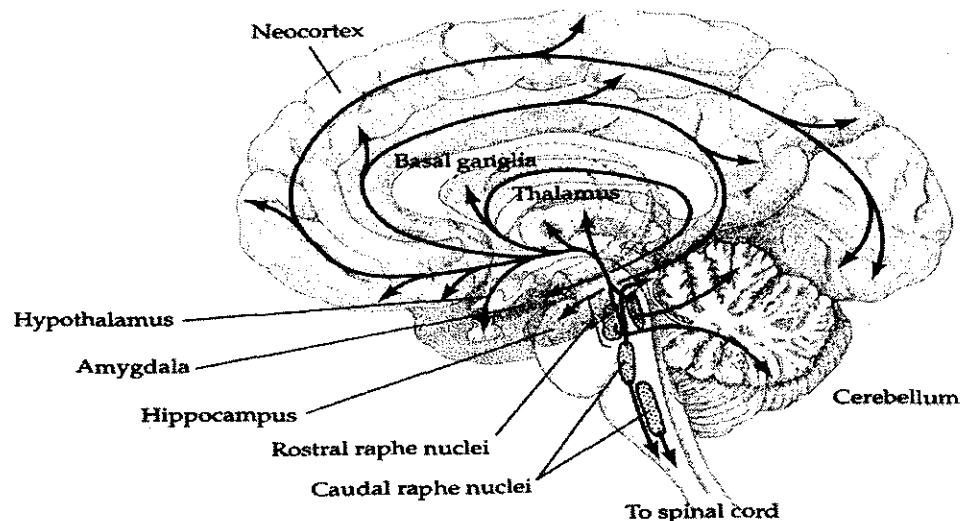


Figure 3.6: Distribution of serotonin in the brain (Nicholls *et al.*, 1992).

Neurons containing serotonin form a chain of raphe nuclei lying along the midline of the brain stem (Nicholls *et al.*, 1992). The serotonergic system consists of two distinct subdivisions: the caudal division and the rostral division (Rang *et al.*, 1999). The caudal nuclei innervate the spinal cord and the rostral raphe nuclei innervate nearly all the regions of the brain (Nicholls *et al.*, 1992) including the forebrain, hippocampus and the cortex (Rang *et al.*, 1999).

3.5.2 Serotonergic receptors

Fourteen different types of 5-HT receptors have been identified (Leonard, 1997). The International Union of Pharmacological Societies (IUPHAR) Commission has classified the various 5-HT receptors according to their ligand binding properties and second messenger systems (Leonard, 1997).

Serotonin receptors have been classified into three main groups (Leonard, 1997):

Those linked to adenylate cyclase via G_i -protein thereby inhibiting the formation of cAMP.

Receptors in this class include the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT₄.

Those linked to the phosphatidyl inositol system, or phospholipase C. The 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} are classified in this group.

Those linked directly to ion channels namely the 5-HT₃.

Activation of the 5-HT_{1A} autoreceptor is responsible for the neuronal inhibition of the firing rate of serotonin neurons thereby inhibiting serotonin release at the nerve terminals. These receptors are abundantly expressed in the dorsal raphe nuclei and the serotonergic cell bodies (Elhwuegi, 2004). 5-HT_{1B} and 5-HT_{1D} autoreceptors alter 5-HT release without altering neuron firing rate (Elhwuegi, 2004).

The 5-HT₃ and 5-HT_{2A} receptors (that are inhibitory in nature) are both not directly involved in 5-HT regulation of neural activity (Elhwuegi, 2004).

3.5.3 Metabolism of serotonin

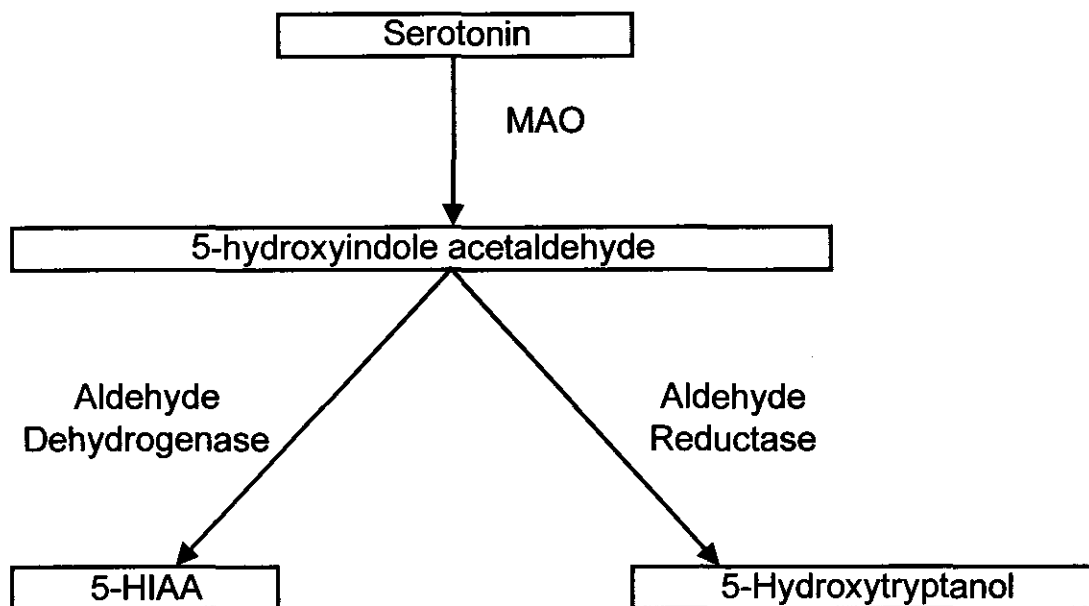


Figure 3.7: Inactivation of serotonin (Adapted from Nicholls *et al.*, 1992).

The principle route of metabolism of serotonin involves monoamine oxidase (MAO) forming 5-hydroxy-indole acetic acid (5 -HIAA) by a two-step process (Figure 3.7)

3.5.4 The role of serotonin in PTSD

Serotonin has been found to have both anxiogenic and anxiolytic properties (Charney *et al.*, 1987).

Increased serotonin activity in the CNS is required for the successful adaptation to stress and the prevention of subsequent learned helplessness (Spivak *et al.*, 1999). Pretreatment of animals with selective serotonin reuptake inhibitors (SSRIs), as well as tricyclic antidepressants before exposure to inescapable stressor, prevents the behavioural syndrome of learned helplessness (Bonne *et al.*, 2003). Learned helplessness is also regarded as a valid model of PTSD (Uys *et al.*, 2003).

Increased hippocampal serotonergic activity has also been observed in chronically stressed animals (Gamaro *et al.*, 2003). Fearfulness is a key symptom of PTSD and might trigger intrusive recollection that in turn will produce or enhance anxiety (van Praag, 2004).

In contrast, decreased serotonin concentrations have been observed in patients diagnosed with combat-related PTSD, providing a possible explanation for the exaggerated startle response

noted in these patients (Spivak *et al.*, 1999). Decreased serotonin has been associated with heightened anxiety, aggression and impaired long-term memory.

An acute increase in 5-HT leads to anxiety followed by anxiolysis during chronic stimulation (Sandford, 2000). This is the result of the initial stimulation of the hyper sensitive receptors followed by down regulation of these receptors in response to chronic bombardment (Sandford, 2000).

Restraint stress has been shown to induce suppression of 5-HT_{1A} receptor binding and atrophy of the dendrites of the CA3 pyramidal neurons (Watanabe *et al.*, 1992). The 5-HT_{1A} receptors exert an inhibitory effect on the hippocampus and increased corticosterone levels decrease this inhibitory effect (Montgomery *et al.*, 2001). Stress induced corticosterone release makes the hippocampus more vulnerable to the laying down of long-term emotion driven memories. The failure to contain the hypersensitive feedback regulation of the HPA-axis function causes a chronic stress response (Harvey *et al.*, 2003).

The 5-HT_{1A} receptor exerts an anxiolytic effect when innervation of the amygdala and hippocampus originating in the median raphe nuclei is mediated by serotonin (van Praag, 2004). Studies have established a regulatory role of serotonin in HPA –axis function, with the 5-HT_{1A} receptor being implicated in particular. Qualitative and quantitative increases in hippocampal 5HT_{1A} receptors induced by the TDS model may account for the disturbance in memory and emotion (Harvey *et al.*, 2003). 5-HT_{1A} receptor density have been found increased on day 7 post RS in a previous study conducted at our laboratory (Naciti, 2002). The serotonin 5-HT_{1A} and 5-HT_{2C} receptor systems exert opposing effects in anxiety regulation.

Kalynchuck and colleagues (2001) have shown that kindled rats have increased hippocampal 5-HT_{1A} receptor density that can be correlated to increased fear. In PTSD it seems that the 5-HT_{1A} receptor system may be both under and over-responsive and both conditions contribute to the symptoms of PTSD i.e. the hyperarousal, fearfulness and reduced habituation to adverse events (van Praag, 2004).

Stimulation of the 5-HT₂ receptors is associated with severe anxiety and an impeded adaptation to stressors (McKittrick *et al.*, 1995). Elevated corticosterone concentrations up regulate the 5-HT_{2A} receptor thereby decreasing serotonin concentrations (Toth & Benjamin, 1997).

Removal of corticosterone by adrenalectomy in animals results in an anatomically specific decrease in 5-HT metabolism while stressful situations increase serotonin turnover (Nishi & Azmitia, 1996).

3.6 Corticosterone (CORT)

Every disturbance in the body evokes a response that serves to restore homeostasis and facilitate adaptation.

Essential to the stress response are the neurons in the paraventricular nucleus (PVN) of the hypothalamus, which expresses corticotropin releasing hormone (CRH) and other neuropeptides that drive the activity of the hypothalamic-pituitary-adrenal (HPA)-axis and the sympatho-adrenomedullary system (De Kloet *et al.*, 1998). Both these systems exert control over each other's function (De Kloet *et al.*, 1998). Of the two systems, the HPA-axis is responsible for the secretion of corticosterone in the rat and cortisol in the human and its action is slower and more persistent.

3.6.1 Synthesis of corticosterone

The synthesis of corticosterone is illustrated in Figure 3.8.

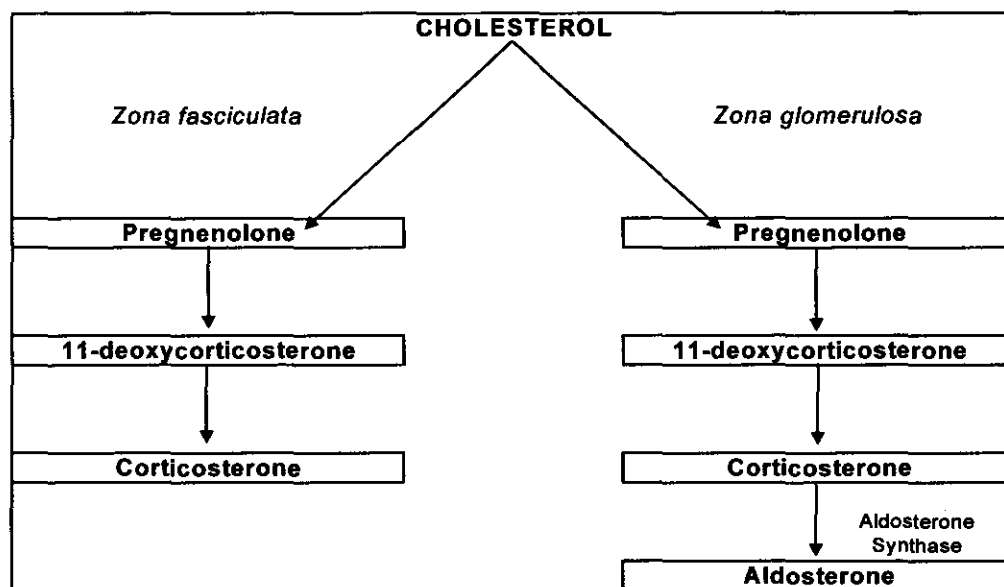


Figure 3.8: Synthesis of corticosterone in the adrenal cortex (Adapted from King, 1999).

Figure 3.8 shows the pathways of corticosterone synthesis. The rate-limiting step is the conversion of cholesterol to pregnenolone. The adrenal cortex uses multiple sources of cholesterol to ensure adequate steroid genesis including circulating cholesterol taken up via the LDL-receptor pathway, liberation of cholesterol from endogenous cholesterol ester stores via cholesterol esterase and increased *de novo* biosynthesis (King, 1999).

The adrenal cortex is composed of 3 main tissue regions: zona glomerulosa, zona fasciculata, and zona reticularis. Although the pathway to pregnenolone synthesis is the same in all zones

of the cortex, the zones are histologically and enzymatically distinct, with the exact steroid hormone product depending on the enzymes present in the cells of each zone. Zona glomerulosa cells are unique in the adrenal cortex in containing the enzyme responsible for converting corticosterone to aldosterone, the principal and most potent mineralocorticoid. The result is that the zona glomerulosa is mainly responsible for the conversion of cholesterol to the weak mineralocorticoid, corticosterone and the principal mineralocorticoid, aldosterone. Cells of the zona fasciculata and zona reticularis lack the C-18 hydroxylase that converts corticosterone to aldosterone, and thus these tissues produce only the weak mineralocorticoid corticosterone (King, 1999).

3.6.2 General mechanism of action of corticosterone

The classical mechanism of action after corticosterone enters the brain involves the binding of glucocorticoid molecules to intracellular receptors (De Kloet *et al.*, 1998). The glucocorticoid receptor (GR) resides predominantly in the cytoplasm in its inactive form until it binds the glucocorticoid ligand. This causes receptor activation and translocation to the nucleus. The inactive GR is found in complex with other proteins including Heat Shock Protein (HSP) 90 a member of the heat shock family of stress induced proteins. HSP 90 is responsible for the folding of the receptor into its appropriate conformation believed to be essential for ligand binding (Schimmer & Parker, 1996).

Following ligand binding, the HSP 90 dissociates and corticosterone is directed into the nucleus where it can interact with specific DNA sequences termed glucocorticoid-responsive elements (GREs). Mineralocorticoid receptor (MR) binding is almost identical, but receptor expressions are restricted to the kidney, colon, salivary and sweat glands and the hippocampus (Schimmer & Parker, 1996).

Corticosterone interacts with specific receptor proteins in target tissues to regulate the expression of corticosteroid responsive genes; thereby changing the levels and array of protein synthesised by the various target tissues (Schimmer & Parker, 1996). Corticosterone is found bound to corticosteroid-binding globulin (CBG), a plasma protein with a high affinity for steroids but low total binding capacity. Only unbound corticosterone may enter cells to mediate an effect (De Kloet *et al.*, 1998).

Access of corticosterone in the brain is controlled by many factors, but considered of significance are the following two factors. The first determinant of access to the brain is CBG. Circulating corticosterone is bound to CBG ($K_d \sim 50$ nM, 4°C). Of the average corticosterone concentration circulating over a 24-h period, less than 5% is not bound to CBG (Smith & Hammond, 1992).

Glucocorticoids and stress down regulate CBG. CBG is thus a static regulator of the biological availability of corticosterone (De Kloet *et al.*, 1998). The second determinant of corticosterone access is a dynamic regulator, the enzyme 11 β -hydroxysteroid dehydrogenase (HSD) (Smith & Hammond, 1992).

Corticosterone displays two modes of action during the stress response- the first being the "Pro-active" mode in which corticosterone maintains and controls basal activity of the HPA-axis and controls the sensitivity of the system's response to stress. It is involved in the co-ordination of circadian events such as the sleep-wake cycle and processes underlying selective attention and response selection (De Kloet *et al.*, 1998).

In the second case it performs in the "Reactive" mode in which corticosterone feedback helps terminate stress-induced HPA-axis activation. The steroid facilitates the animal's ability to cope with, adapt to and recover from stress. Corticosterone also promotes learning and memory processes (De Kloet *et al.*, 1998).

There is growing evidence that glucocorticoid activity is also mediated by a higher order of control of receptor interaction with the genome by a rapid non-genomic mechanism that is poorly understood. This conclusion was drawn by indirectly assessing the rapidity of effect, resistance to protein synthesis inhibition and receptor blockade (Mikics *et al.*, 2004).

In general, effects occurring in less than 15 minutes and which are not blocked by protein synthesis inhibition or by receptor blockers are attributed to non-genomic mechanisms (Mikics *et al.*, 2004). Additional evidence involves glucocorticoid effects exerted in a genome-free environment or by glucocorticoid protein conjugates that do not penetrate the cell to reach the cytoplasmic receptors (Chen *et al.*, 1991).

The rapid, non-genomic effects of glucocorticoids may constitute a potentially important factor of behavioural response to challenges (Mikics *et al.*, 2004). Research has shown that high levels of corticosterone over long periods of time injure the hippocampus causing memory disturbance and the same is true for very low cortisol levels (Sapolsky, 2000). Corticosteroid concentrations in the middle range are regarded as adaptive and maintaining corticosterone concentrations in this range is essential for a successful response to stress (van Praag, 2004).

3.6.3 Corticosteroid receptor diversity

The actions of corticosterone in the brain are mediated by glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). These two separate but related intracellular receptors for adrenal steroids have been characterised by receptor binding studies (De Kloet *et al.*, 1998). If

these receptors are co-localised in the brain both synergistic and antagonist effects may be exhibited.

Table 3.1: Two intracellular corticosteroid receptors in the brain (De Kloet *et al.*, 1998).

<u>Mineralocorticoid Receptor (MR): Type I</u>	<u>Glucocorticoid Receptor (GR): Type II</u>
☐ High affinity for corticosterone ($K_D \sim 0.5\text{nM}$) ☐ In limbic brain structures	☐ Lower affinity for corticosterone ($K_D \sim 5\text{nM}$) ☐ Ubiquitous

Thus the brain contains two receptor systems which both bind corticosterone but with a difference in the affinity. The type I receptor also known as the mineralocorticoid receptor (MR) because of its high affinity for aldosterone, has an equally high affinity for corticosterone, the primary endogenous glucocorticoid of the rat.

One of the consequences of the very high affinity of the MR is that these receptors are more than 80% occupied by the endogenous hormone even under basal resting conditions. The occupancy of the GR varies in parallel with changes in plasma corticosterone levels during circadian variation or following stress (De Kloet *et al.*, 1998).

The type II receptor also known as the glucocorticoid receptor (GR) has a 6 to 10-fold lower affinity for corticosterone (De Kloet *et al.*, 1998). Type II receptors are more ubiquitous and are present in moderate amounts in the brain, neurons that are richly endowed with GR are those associated with the regulation of the stress response for example (De Kloet *et al.*, 1998). These receptors are occupied during times of higher circulating levels of corticosterone and suppress subsequent HPA-axis activation in times of stress (McEwen *et al.*, 1986).

Anatomically the hippocampus has received much attention, given that both MR and GR are found in high concentrations here (Sapolsky *et al.*, 2000). Type I receptors are especially abundant in the brain but its location is limited to the periventricular areas, the nucleus tractus solitarius (McEwen *et al.*, 1986). This receptor is also present in the kidney.

This site dependent specificity of the mineralocorticoid receptor has been linked to the enzyme 11 β -hydroxysteroid dehydrogenase and corticosteroid-binding globulin (CBG, transcortin),

which protect the MRs in the kidney from excessive stimulation by the endogenously occurring glucocorticoids (Funder *et al.*, 1988).

The diversity of the effects suggests that a balance in MR and GR mediated effects exerted by corticosterone are essential for homeostatic control. In relation to PTSD the GRs and MRs play different roles. The MRs are responsible for selective attention, integration, interpretation and appraisal of novel information and for the execution of an appropriate behavioural response. Understimulation of these receptors due to a subnormal cortisol concentration would impair ones ability to adjust. The GRs are responsible for the processes leading to the storage of information and the elimination of a maladaptive response (van Praag, 2004).

3.6.4 The role of corticosterone in PTSD

Exposure to an acute stressful event leads to the rapid release of corticosterone (Kanter *et al.*, 2001). This increase in corticosterone level is necessary for the establishment of homeostasis and consolidation of the information associated with the event in order to maximise the likelihood of survival in the case of a re-encounter (Korte, 2001).

PTSD differs from other anxiety disorders in that it can be associated with below basal corticosterone levels (Kanter *et al.*, 2001). Some studies have reported a peripheral hypocortisolism (Naciti, 2002; Oosthuizen, 2003). This decrease in cortisol levels in the aftermath of a stressful event can be ascribed to a hypersensitive negative feed-back mechanism and a sensitisation of the inhibitory elements of the HPA-axis (Liberzon *et al.*, 1997).

Low levels of cortisol impair memory functions essential for an effective stress response (Sapolsky, 2000) thereby impairing proper appreciation of the actual situation. This makes the differentiation of relevant and irrelevant information difficult, and impedes the capacity to adjust appropriately. Under stimulation of MRs has been attributed to behavioural abnormality (van Praag, 2004).

The role of corticosterone has extensively been studied (Korte, 2002): basal levels of corticosterone, occupying hippocampal mineralocorticoid receptors (MRs), enable the expression of the unconditioned fear response (4.3.1). Increased corticosterone levels then act through glucocorticoid receptors (GRs) in the acquisition of both cue and context conditioned fear response.

Once aversive conditioning has been established, corticosterone, via MRs, exerts a permissive action for the expression of the immediate conditioned fear response, after exposure to a

stressor. Increased corticosterone brain GR-occupation then promotes processes underlying generalized fear response. Binding of corticosterone to brain MRs is a prerequisite for the extinction of passive avoidance behaviour, whereas GRs are involved in the extinction of active avoidance behaviour. Potentiation of fear response appears to be dependent upon GRs. Thus, MRs and GRs are jointly involved in the different stages of associative learning.

Other studies do not support the concept of either a static "hypo/hyper-cortisolism" in PTSD, but rather suggest a psychogenic basis for cortisol alterations in PTSD in relation to psychosocial stress. This indicates a central regulatory dysfunction of the hypothalamic-pituitary-adrenal axis. This is characterised by a dynamic tendency to overreact in both upward and downward directions (Mason *et al.*, 2002).

Souza and De Loon (1986) have found an inverse relationship between plasma corticosterone concentrations and 5-HT turnover in the CNS. Decreased cerebrospinal 5-HIAA concentrations positively correlate with anxiety since 5-HIAA is to a large extent a function of serotonin metabolism (van Praag, 2004 a).

While the hippocampus regulates glucocorticoid release through its inhibitory actions on the HPA-axis (Brenner, 1999), hippocampal damage would result in the disruption of the negative feed-back loop, thereby making the hippocampus increasingly vulnerable to the toxic effects of glucocorticoids (Nutt, 2000). Decreased hippocampal volume have been reported to in patients with PTSD. This has been ascribed in part to increased exposure of the hippocampus to corticosterone (Sapolsky, 2000). It is possible that the massive secretion of corticosterone upon exposure to the trauma is the first step in a cascade of events leading to a maladaptive HPA-axis, response leading to hippocampal atrophy and subsequent memory deficits in PTSD.

Glucocorticoids are reported to decrease glucose uptake and cellular energy. This could eventually lead to decreased cellular metabolism ultimately resulting in neurotoxicity (Duman *et al.*, 1999). Another mechanism thought to be involved in the actions of stress and glucocorticoids is increased glutamate and calcium excitotoxicity.

Stress and administration of glucocorticoids are reported to increase glutamate levels. Glutamate acting via NMDA receptors increases intracellular levels of calcium ions. Sustained glutamate-induced calcium release is known to contribute to glucocorticoid-induced neuroendangerment (Duman *et al.*, 1999).

3.7 Interactions between the DA, NA, 5-HT & corticosterone

Numerous studies have suggested a bi-directional interaction between corticosterone and the monoamines in the brain (see **Figure 2.2**) at cellular and sub-cellular levels.

The capacity for cellular homeostasis in the monoaminergic systems is regulated by the fundamental ability to adjust the level of monoamine synthesis and release as a function of ongoing activity requirements (Abercrombie & Zigmond, 2000).

Neurotrophic factors were originally characterised based on their ability to influence the differentiation and development of specific population of neurons in the CNS (Duman *et al.*, 1999). BDNF belongs to the neurotrophic factor family that can be induced in response to neural activity. It has been shown to play a critical role in cellular models of learning and memory, particularly in long term potentiation (Duman *et al.*, 1999).

The mechanism underlying the influence of stress on BDNF expression has not been fully characterised. It has been suggested that down regulation of BDNF can be attributed to elevated corticosterone levels (Duman *et al.*, 1999). Smith (1996) reported that elevated corticosterone levels are responsible for the decreased expression of BDNF, even in the absence of the adrenal gland. Another possibility is that noradrenergic and serotonergic systems, are activated by stress and thus contribute to decreased BDNF expression. It has been suggested that BDNF could influence the monoaminergic system actions by increasing the function of monoamine neurons or increasing the output of target neurons (Duman *et al.*, 1999).

Functional receptor interactions, between different receptors within a single neuron, via second messenger systems act as co-regulators of cell function (Harvey, 1997). The expression of cAMP-dependent protein kinase (PKA) and cAMP response binding element (CREB), a transcription factor that mediates many effects of cAMP on gene expression is found up-regulated by antidepressant treatment (Duman *et al.*, 1999). Antidepressants target multiple MA systems.

CREB may be phosphorylated by PKA via NA and 5-HT receptors and by other receptors/ion channels that influence calcium ion signalling (Duman *et al.*, 1999). The expression and activation of CREB is particularly interesting since various transduction pathways can activate it to influence neuronal function and behaviour in the CNS.

It is postulated that D₁ receptors linked to the PKA pathway via G-proteins increase PKA activity (Birnbaum *et al.*, 2000), suggesting that activation of the cAMP/PKA can in part be held responsible for the memory deficits caused by stress. This hyperactivity results in lower aminergic activity in the prefrontal cortex. Desensitisation of the α_2 receptors may increase the sensitivity of the stimulatory effect of the α_1 receptor thus increasing the release of 5-HT and NA at certain synapses in the brain.

The efficacy of both fluoxetine (SSRI) and bupropion in the treatment of PTSD involve interaction between NA, DA and 5-HT. In vivo studies have indicated that exposure of the nucleus accumbens to serotonin results in increased dopamine release (Sasaki-Adams & Kelley, 2001). The actions of bupropion, a commonly used antidepressant although not clear, is thought to be mediated via noradrenergic and dopaminergic effects (Sasaki-Adams & Kelley, 2001). These findings suggest synergy between the monoaminergic systems.

Cognitive functions such as working memory, behavioural inhibition and attention regulation rely on the integrity of the PFC (Birnbaum *et al.*, 2000). Experimental depletion of NA and DA using the catecholamine neurotoxin, 6-hydroxydopamine (Arnsten, 1998) resulted in decrements of PFC function, suggesting that these monoamines regulate PFC activity (Birnbaum *et al.*, 2000).

Administration of reserpine, which caused a non selective monoamine depletion impaired memory (Castellano *et al.*, 1996). This impairment was reversed by the administration of DOPA (Castellano *et al.*, 1996), a precursor of DA.

Stress induced working memory deficits has also been reversed by the systemic pre-treatment with a dopamine antagonist (D₁ receptor) clozapine (Murphy *et al.*, 1997), or the local infusion of a α_1 receptor antagonist (Birnbaum *et al.*, 2000). It has been shown that depletion or excessive DA or NE levels are detrimental to memory (Birnbaum *et al.*, 2000).

Elevation of extracellular NA levels by the local administration of desimipramine significantly increased extracellular DA concentrations and strongly potentiated stress induced DA release. This effect may be due to the stimulatory effect of NA on pre-synaptic DA release or the fact that a relatively larger proportion of DA was taken up in comparison to NA (Tzschenke, 2001).

Enhanced monoamine synthesis during chronic stress may protect against the depletion of stores, permitting an organism to respond more effectively to a novel stressor (Abercrombie & Zigmond, 2000). The mechanism by which corticosterone facilitates the MA release is still not clear. Three possible mechanisms have been suggested: tyrosine hydroxylase activity is increased by corticosterone thereby increasing MA synthesis, or inhibition of MAO activity by corticosterone or decreased dopamine and noradrenaline re-uptake (Piazza & Le Moal, 1997).

Removal of corticosterone by adrenalectomy results in anatomically specific decrease in 5-HT metabolism while stressful procedures increase serotonin turnover (Nishi & Azmitia, 1996). The normal rise in CORT levels in response to a stressor, is accompanied by an immediate release of plasma NA levels followed by a transient decrease in NA levels (Laakman *et al.*, 1984).

Since the LC also indirectly stimulates the HPA-axis via the limbic system (De Bellis *et al.*, 1999), an increased NA concentration following exposure to a stressor is expected. When a normal rise in corticosterone is prevented by an adrenalectomy, acute stress results in an even greater increase in plasma NA concentrations (Brown & Fisher, 1986). It is proposed that stress induced corticosterone release allow for better coping response.

Elhwuegi (2004) explains that changes in MA levels in the CNS make the central α_2 auto and hetero-receptors hypersensitive. The α_2 autoreceptors are linked to cAMP/protein kinase A (PKA) and thus the activation of these receptors can decrease PKA activity (Birnbaum *et al.*, 2000). Stimulation of the post-synaptic α_2 autoreceptor enhances PFC activity and may improve behavioural performances of animals with depleted/excess NA levels (Birnbaum *et al.*, 2000).

The release of both NA and 5-HT is regulated by the α_2 noradrenergic receptor on the noradrenergic neuron (autoreceptor) and the 5-HT neurons (heteroreceptor), resulting in the attenuation of noradrenaline and serotonin release (Leonard, 1997). Serotonergic pathways make synaptic connections via heteroreceptors with dopaminergic and noradrenergic (Leonard, 1997) pathways, thus the function of dopamine and noradrenaline is modulated by serotonin.

Activation of the 5-HT_{1A} receptors stimulates dopamine release in the nucleus accumbens (Dremencov *et al.*, 2004) and this area of the brain is associated with motivation. Desensitisation of the 5-HT_{1A} and 5-HT_{1B} autoreceptors may possibly restore normal function of the CNS (Elhwuegi, 2004). Down regulation of the 5-HT_{1A} autoreceptors and the α_2 noradrenergic receptor is implicated in the disinhibition of serotonin release (Harvey, 1997).

The 5-HT_{1A} receptors exert an inhibitory effect on the hippocampus and increased corticosterone levels decrease this inhibitory effect (Montgomery *et al.*, 2001). Increased corticosterone levels, also selectively increase 5-HT_{2A} receptor activity in the PFC (Katagiri *et al.*, 2001). This receptor has been implicated in playing a role in anxiety. Activation of the 5-HT_{2A} receptors further activates the HPA-axis (Heimrick-Leucke & Evans, 2002) and stimulates the release of ACTH and corticosterone. Pre-clinical data suggests that the antagonism of the 5-HT_{2A} receptor decreases both the behavioural and physiological response to stress (Kent *et al.*, 2002).

PTSD comprises a combination of numerous interrelated neurobiological mechanisms that argue the pathological response to stress. This study attempted to clarify the possible links if any, between the behavioural, biochemical and neuroendocrine correlates in rats after exposure to the stressors of the TDS model.

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Animal Models of PTSD and Anxiety

Chapter 4

4.1 Introduction

The range of behaviour that has been suggested to reflect emotional states in experimental animals is large. Clinical studies suggest that some individuals exposed to severe stress develop long-term changes in specific brain systems (Bremner *et al.*, 1991). Animal models of stress have the potential to provide information on the course and etiology of PTSD since they offer the possibility of stimulating a human condition under controlled circumstances, with a large number of subjects, in a simpler more readily understandable system (Yehuda & Antelman, 1993).

Animal models of psychiatric disorders have been useful in clarifying the connection between biological changes and behavioural abnormality. In the case of PTSD there is greater potential to accurately model the disorder since the major precipitating factor (response to severe traumatic situation) is known (Yehuda & Antelman, 1993).

4.2 Animal Models of PTSD

In considering relevant animal models of PTSD it is critical to differentiate between factors that influence the manifestation of PTSD and those that are essential for induction. Yehuda and Antelman (1993) have derived the following criteria for evaluating the relevance of individual stress paradigms of the phenomenology of PTSD. These criteria were derived by pairing down PTSD to its most basic components. The four identified criteria are:

- 1.) *The stressor should be capable of producing PTSD-like symptoms in a dose-dependent manner.*** Any type of stressor of sufficient magnitude should be able to induce the biobehavioural sequel of PTSD. Therefore, although duration of the stressor does not seem to be directly relevant to predicting the clinical and, possibly, the biological consequences of PTSD, the intensity of the stressor is relevant to this issue. There is evidence for a relationship between stressor intensity and severity of PTSD symptoms. Moreover, a useful animal model for PTSD would be one in which behavioural and biological sequel of the stressors have been studied in a dose-dependent manner and in which changes in relevant biological systems show differential responsivity to different levels of similar stressors (Yehuda & Antelman, 1993).

- 2.) The stressor should produce biological alterations that persist over time or become more pronounced with the passage of time.** Given that the onset of PTSD is often delayed for months or even years in many individuals, a gradual worsening of biological abnormalities with the passage of time would be expected. A good animal model of PTSD therefore, would theoretically allow for the possibility of recovery or attenuation of symptoms in addition to relapse or late onset. It has been well documented that several biological systems are altered immediately following stress, however, many of these systems show a rapid recovery from stress. For this reason, paradigms in which animals are only studied immediately following exposure to stress do not produce information directly relevant to the biology of PTSD unless they are also followed by long-term studies (Yehuda & Antelman, 1993).
- 3.) The stressor should induce biobehavioural alterations that have the potential for bi-directional expression.** The core symptoms of PTSD include manifestations of both enhanced and reduced responsiveness to environmental stimuli that recall the initial trauma. Therefore, an animal model of PTSD should exhibit the capacity for bipolar, or bi-directional manifestations through alternations between excitatory and inhibitory biobehavioural changes (Yehuda & Antelman, 1993).
- 4.) Inter-individual variability in response to a stressor should be present either as a function of experience, genetics or an interaction of the two.** Several factors other than the original trauma may contribute to the induction of PTSD. The observation that not all persons who are exposed to extreme trauma develop symptoms has prompted many studies to examine the effect of prior stress history, family studies etc (Yehuda & Antelman, 1993).

Animal reactions to a recognisably life-threatening situation may be particularly relevant to PTSD. Van der Kolk (1998) stresses the importance of gaining knowledge of animal response to life-threatening situations in order to understand the biological adaptations of people to inescapable stress.

From the onset it is important to realise that no animal model, natural or induced, can ever fully mirror the human situation it is modelling. This difficulty according to Smith (1965) may be due to (1) intrinsic properties with defining patient profile and diagnostic criteria, (2) inter-patient variation, (3) the level that the animal displays may not be at the level that is most pathological. Even when behaviour and signs are the same it should be remembered that humans are

unusual among animals in that they are verbal and thus similar behaviours, signals and signs may have different meanings.

Many animal models capable of inducing PTSD-like symptoms using a large variety of stressors have been validated and reviewed in the literature. A stressor can be defined as an event that endangers or is perceived to endanger the survival of an individual (van de Kar & Blair, 1999).

Stressors can be categorised into three broad categories: a) psychological stressors that are based on a learned response to a threat of a impending adverse condition (fear, anxiety, exposure to an uncontrollable environment); b) stressors that consist of a physical stimulus and have a strong psychological component (pain, immobilisation) and c) stressors that challenge the cardiovascular homeostasis (heat exposure, exercise) (van de Kar & Blair, 1999).

Examples of these models used to induce PTSD-like symptoms include electric shock, learned helplessness (LH), stress-restress or time dependent sensitisation (TDS), under water trauma and exposure of rats to a predator (Uys *et al.*, 2003). Of these models the **Learned Helplessness (LH) model** (Yehuda & Antelman, 1993) and the **Time Dependent Sensitisation model (TDS)** (Liberzon *et al.*, 1997) are the most familiar. It should again be re-emphasised that these models play a pivotal role in investigating the mechanisms underlying anxiety-related disorders such as PTSD. For the purpose of this study a previously validated **Time Dependent Sensitisation model (TDS)** was used (Harvey *et al.*, 2003).

4.2.1 The Learned Helplessness (LH) Model

This model selectively utilises an unpredictable, uncontrollable stress event to determine individuals with an escape deficit in subsequent controllable stress paradigms. Researchers have used this model to better understand the possible neurobiological factors underlying PTSD. This model was initially proposed as a model of depression, since findings of noradrenaline depletion and behavioural passivity appeared to reflect the clinical aspects of this disorder. It is an accepted model of PTSD (Krystal *et al.*, 1989) despite the fact that depression and PTSD have been shown to be clinically and biologically different.

4.2.2 Time Dependent Sensitisation (TDS) Model

The behavioural model of stress-restress or TDS has been proposed as a useful model for PTSD (Yehuda & Antelman, 1993). Using this model findings have reported changes in a) neurotransmitter and endocrine systems (Antelman *et al.*, 1991), b) the immune system (Antelman *et al.*, 1990), c) cardiovascular system (Antelman *et al.*, 1989) and d) carbohydrate metabolism (Antelman *et al.*, 1990).

TDS refers to the exposure of a rat to an acute intense stressor. For example restraint stress can induce long lasting alteration in the organism (Yehuda & Antelman, 1993). In this paradigm rats receive one exposure to an inducing stressor and are later tested on the same or another recall stressor. The rationale being that the recall-stressor contributes to the fear related behavioural disturbance over time (Uys *et al.*, 2003). Physiological and behavioural responsivity to the second stressor differs from those receiving the stressor for the first time.

In a review by Yehuda and Antelman (1993), it was concluded that the TDS model was a promising behavioural model for the induction of PTSD-like sequel in experimental animals. Different models have shown reliability in causing stable, long-term changes after brief but intense stressful events. These alterations are noted in behavioural reactivity, hormonal and autonomic functions (Stam *et al.*, 2000). Of considerable importance is the type of stressor used for sensitisation and these may differ as painful, social or physiological stimuli (Stam *et al.*, 2000).

The use of restraint as a stressor has been proven to induce stress in rats and this has long-term effects (Dal-Zotto *et al.*, 2003). Its effects are bi-directional, which reduces both central and peripheral HPA responses (Dal-Zotto *et al.*, 2003). This has important consequences for the study of PTSD. **Swim stress** also acts as an unpredictable, uncontrollable stress event that has the capability to induce change in activity and dysregulation of the HPA activity (King *et al.*, 2001).

A study done by Cordeo and co-workers (2003) showed that contextual fear conditioning by a previous acute stressor was not maintained at one week post training. In the TDS model, following the acute stressor rats are exposed to a mild situational reminder (RS) on day 7. The TDS model emphasizes the role of past trauma in predicting subsequent dysfunction, provides credible intrasubject variation and allows for either enhanced or reduced responsiveness to environmental stimuli (Yehuda & Antelman, 1993).

It has proved useful for studying hypothalamic-pituitary-adrenal abnormalities relevant to PTSD (Liberzon *et al.*, 1997). Animals subjected to the TDS show enhanced sensitivity to the negative glucocorticoid feedback that is characteristic of PTSD while displaying distinct changes in hippocampal glucocorticoid and mineralocorticoid receptor expression (Liberzon *et al.*, 1999).

4.3 Methods of inducing anxiety

Associative learning can account for the development of an emotional response. The development of fear can simply be seen as a consequence of the association of an event or stimulus with an unpleasant experience. Such methods are referred to as Pavlovian conditioning methods and are regularly used to induce stimulus specific fear in laboratory animals (Cardinal *et al.*, 2002).

4.3.1 Pavlovian conditioning

Pavlovian or classic conditioning refers to a set of experimental procedures in which the experimenter arranges a contingency between stimuli in the world by presenting those stimuli independent of an animal's behaviour. It is also known as fear conditioning and makes no assumption as to what is learned (Cardinal *et al.*, 2002).

It is a neural mechanism that involves the amygdala, locus coeruleus, hippocampus and thalamus (Rosenweig *et al.*, 1996). Fear conditioning may account for the common clinical observations in PTSD patients that elicit symptoms such as anxiety and hyper-arousal resulting in the frequent re-experiencing of the trauma (Cardinal *et al.*, 2002).

In this type of study (Figure 4.1), an initially neutral stimulus is paired with a biological relevant unconditioned stimulus that normally elicits an unconditioned response. As a result of this pairing the unconditioned stimulus becomes a conditioned stimulus and the unconditioned response becomes a conditioned response (Cardinal *et al.*, 2002). Pavlovian conditioning allows the animal to predict events occurring in the environment and thus to adapt to different situations.

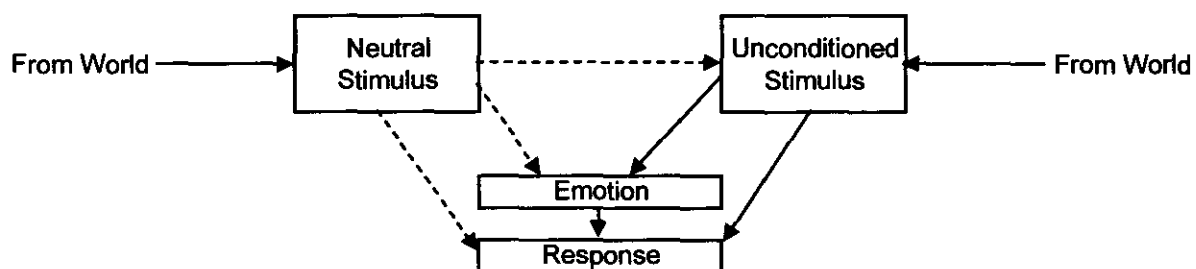


Figure 4.1: The concept on which Pavlovian conditioning is based (Adapted from Cardinal *et al.*, 2002).

The conditioned stimulus is now capable of evoking an affect such as fear (Cardinal *et al.*, 2002). Contextual information requires the hippocampus to first create a representation of the environment utilising information received from the entorhinal cortex and subiculum. This information is then relayed to the amygdala (Sullivan *et al.*, 1999).

The central nucleus of the amygdala is considered critical in the expression of the conditioned fear response as it projects to the paraventricular nuclei of the hypothalamus and these nuclei are involved in the activation of the HPA-axis (Sullivan *et al.*, 1999). The amygdala is involved in the pavlovian conditioning of emotional responses (Cardinal *et al.*, 2002). Normal fear responses are adaptive in that they serve as protection from potential harm. With repeated exposure, fear can become sensitised so that it eventually results in behavioural responses that are maladaptive. One such response is the kindling theory (Kalynchuk & Meany, 2003).

4.3.2 Kindling / sensitisation

This refers to a phenomenon whereby repeated excitation or arousal results in a progressive intensification of responses even when exposed to mild stimuli. Kindling results in the permanent sensitisation of brain circuits such as the hippocampus and other limbic areas that are responsible for behavioural manifestations of fear (Kalynchuk *et al.*, 2001). Rats that have become kindled are unable to properly process contextual information of the stimulus and therefore cannot switch-off the stress response. They display anxiety on various behavioural models such as the **Elevated Plus Maze (EPM)** model (Kalynchuk & Meany, 2003).

The development of fear in kindled rats may be related to decreased GR mRNA expression in the dentate gyrus and CA1 subfield of the hippocampus. Because hippocampal GR are critically involved in negative feedback control of the HPA axis, findings suggest that kindled rats may experience a stronger and longer-lasting HPA axis response to a stressful stimulus than normal rats (Kalynchuk & Meany, 2003).

The effect of trauma in the induction of PTSD-like symptoms can be summarised in **Figure 4.2**.

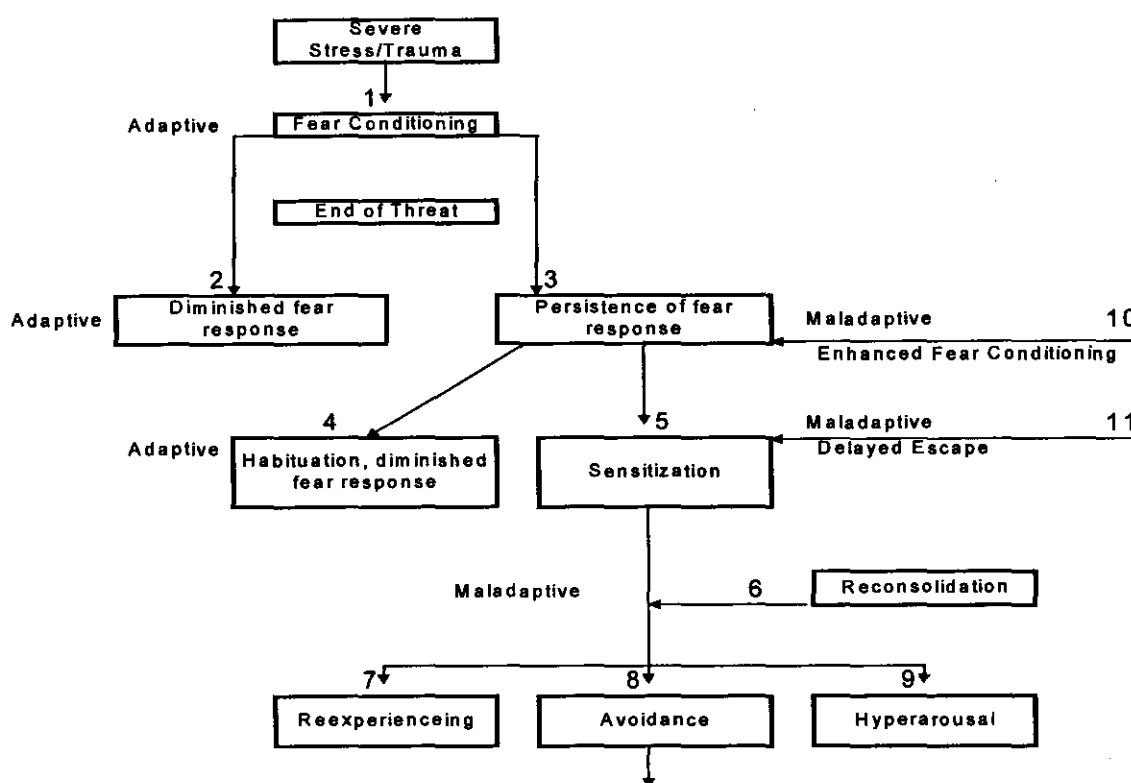


Figure 4.2: Adaptive and maladaptive responses of rodents exposed to extreme stressors for the induction of PTSD-like symptoms (Adapted from Bonne *et al.*, 2003).

According to **Figure 4.2**, exposure to a severe traumatic event (1) results in fear conditioning that serves an adaptive purpose as long as a threat is present. After termination of danger, fear response is normally reduced (2). However, sometimes fear response persists despite termination of the threat (3). Hypothesized mechanisms that promote maladaptive fear conditioning are incubation, hyper-mnesia and enhanced recall, deficit in integration of context and content and impaired alternative learning. Fear response may then habituate and lose its aversive properties (4), or maintain and even augment them (5). Fear response often generalizes, and is evoked by stimuli only remotely similar to initial trauma, reflecting sensitization and cross sensitization of the neuronal system (6). All DSM-IV PTSD symptom clusters are observed at this stage: Reexperiencing (7), Avoidance (8), and hyperarousal (9), reflecting incessant anxiety and absence of a safe haven, generalization and cross sensitization of the conditioned fear stimulus. Such non-associative learning processes increase baseline anxiety and result in full-blown fear responses, exacerbating the reexperiencing cluster. This cascade is positively reinforced by enhancement of fear conditioning (10) and delayed escape behavior (11) that are part of the learned helplessness syndrome.

The Diagnostic and Statistical Manual of Psychiatric Disorders (DSM IV) classifies PTSD as an anxiety disorder (APA, 1994). It is therefore of vital importance that the animal models used to mirror PTSD-like symptoms should be capable of inducing anxiety. Anxiety can be detected on models that monitor behavioural change that reflects increased aversive behaviour.

4.4 Animal Models of Anxiety

Symptoms of anxiety appear when the information perceived by the individual and memories from a past experience mismatch (Beuzen & Belzung, 1995). Brain structures involved in the regulation of emotion and memory are involved in the neurobiology of anxiety disorders.

The pons, medulla and midbrain are responsible for the physiological correlates of anxiety such as the fight-flight reaction (Sandford *et al.*, 2000). The locus coeruleus is a noradrenergic nucleus with efferents to the cortex and limbic areas of the brain. It is implicated in the alarm reaction and is activated during stress (Sandford *et al.*, 2000). The hypothalamus receives input from the limbic system and the locus coeruleus. The hypothalamic-pituitary-adrenal axis (HPA-axis) is activated during stress and is thus an important common pathway in the mediation of anxiety responses (Sandford *et al.*, 2000).

The amygdala is involved in the processing of fear relevant information (Sullivan *et al.*, 1999) and the hippocampus and pre-frontal cortex are responsible for the perceived relaying of fear-inducing stimuli (Rosenweig *et al.*, 1996).

There are two classes of animal behaviour models used for determining anxiety in rodents: a) **conditioned** and b) **unconditioned** (Rodgers & Dalvi, 1997).

Conditioned models involve considerable training of subjects (rodents, pigeons, etc), food or water deprivation and/or the use of electric shock as an aversive stimulus. Models that are based on conditioned behaviour involve responses controlled by operant conditioned behaviour, an example being the fear-potentiated startle response (Oliver *et al.*, 2000). A symptom of psychoemotional disturbance related to fear and anxiety is the enhanced startle response. Exaggeration of the startle response is a criterion of PTSD. The startle is easily quantified as the muscular contraction in response to a sudden unexpected environmental stimulation (Maslova *et al.*, 2002). Habituation of the startle response is observed after repeated signal presentation. Freezing, in which the rat remains motionless is also indicative of fear in the rodent (Cardinal *et al.*, 2002).

Unconditioned models study response to external threats, which rely on the natural behavioural reactions, and do not require specific training. Examples include the elevated plus

maze (EPM), light-dark box exploration and social interaction. Often the latter models are ethologically based, where the model is species-specific (Oliver *et al.*, 2000). Some of the models used in the determination of anxiety are discussed shortly, later in the chapter. In our study unconditioned behaviour was examined using the EPM due to apparatus availability at our laboratory.

4.4.1 The Elevated Plus Maze Procedure (EPM)

4.4.1.1 Origin and early development

Investigators have suggested that human and animal anxiety states differ along cognitive and behavioral lines respectively (Wall & Messier, 2001). This notion is understood on the premise that anxiety is a mental state in the human (Clement & Chapouthier, 1998) but a very basic emotion (fear) in the rodent (Montgomery, 1955).

The Elevated Plus-Maze test is probably the most popular of all currently available animal models of anxiety since it offers an excellent example of a model based on the study of unconditioned behaviour (Dawson & Trickelbank, 1995).

In a study to investigate whether novel stimulation evokes fear as well as exploratory behavior in the rat, Montgomery (1955) reported that open elevated alleys evoked greater avoidance than closed alleys. This model was redefined (Handley & Mithani, 1984) and has extensively been validated pharmacologically, physiologically and behaviorally (Pellow *et al.*, 1985).

Either forced or voluntary entry into the open arms is accompanied by elevated corticosterone levels, increased freezing and production of fecal boli, hormonal and behavioral changes all of which are an indication of anxiety (Pellow *et al.*, 1985).

Normal exploratory behavior is to stay in the closed arms, and this tendency to stay in the closed arms can be increased by using compounds that increase the aversion towards the anxiety provoking open arms. In contrast anxiolytic compounds reduce natural aversion of the open arms and promote exploration thereof (Pellow *et al.*, 1985).

The critical determinants which are therefore correlated with anxiety are the number of entries made into the open arms and the time spent in the open arms (Lister, 1987).

Commenting on the merits of the Elevated Plus-Maze, Pellow and colleagues (1985) state that this test is::

- Fast.

- Simple.
- Does not involve expensive equipment or extensive training and
- is bi-directionally sensitive to manipulations of anxiety.

However the EPM is subject to many methodological variations between laboratories and this may be the root cause of inconsistent findings (Griebel *et al.*, 1993).

4.4.1.2 Methodological variables

Rodgers and Dalvi (1997) believe that the relevant factors may be classified under three headings: organismic, procedural and scoring technique variables. The former two categories impact upon behavioral baselines and the latter upon behavioral specificity.

The principal organismic variables of interest are species, strain, age and gender. Rats and mice have been the predominant species used in Elevated Plus-Maze research (Rodgers & Dalvi, 1997).

Much inter-species variation has been reported in rats (Pellow *et al.* 1985). Anxiety increases with age and gender. Housing conditions, lighting levels, pre-test handling, time and duration of testing all influence the basal levels of anxiety (Hogg, 1996).

Critically important could be the construction of the maze. The addition of ledges around the open arm influences the component of anxiety to which the apparatus is sensitive. The predisposition of the animal to be anxious may provide more insight into the mechanisms involved in mediating anxiety per se (Hogg, 1996).

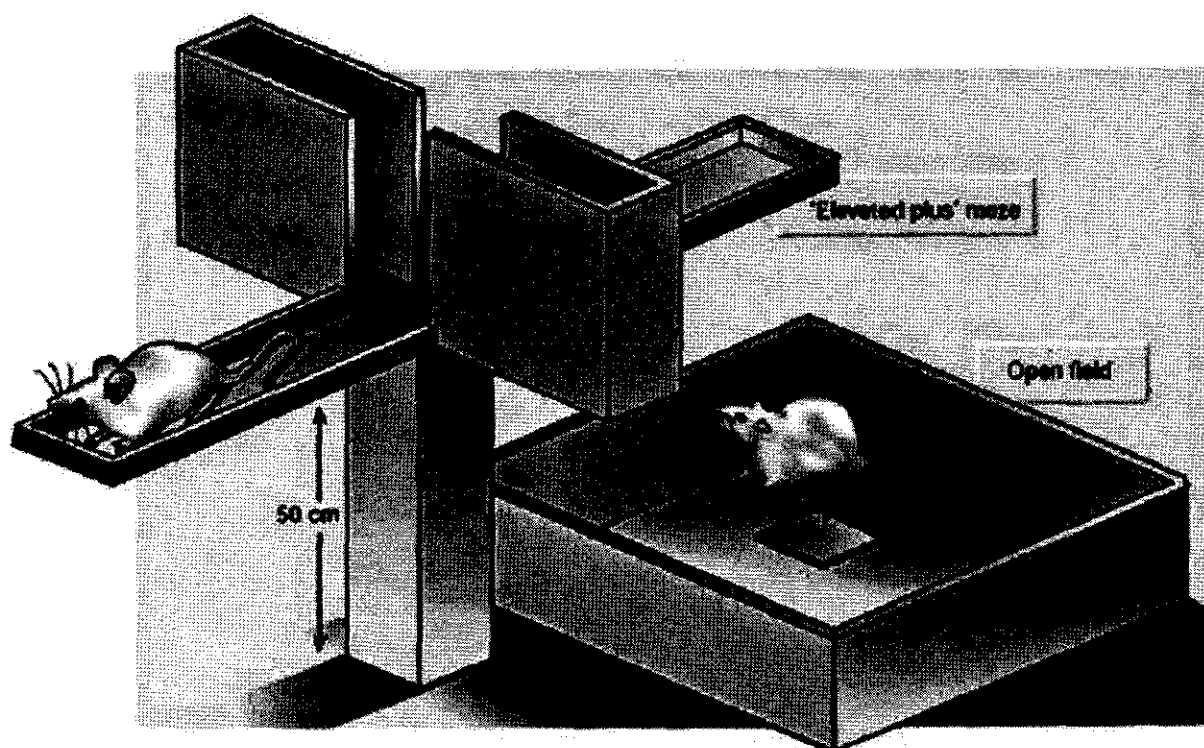


Figure 4.3: Structure of the Elevated Plus Maze and the Open field apparatus.

4.4.1.3 The utility of the EPM in anxiety detection

Exposure of rodents to stressors influences behaviour on the EPM, though reports have been inconsistent. Acute stressors such as forced swim and immobilisation have been shown to decrease open arm exploration (Hogg, 1996; Korte & De Boer, 2003). Partial immersion in water for 15 minutes did not influence % time in open arms (Korte & De Boer, 2003).

Korte & De Boer, (2003) found that exposure to predator stress maintains an enhanced anxiety state for as long as 3 weeks post exposure, while Adamec and co-workers (2004) reported that exposure to predator stress has no effect on both open and closed arm exploration.

Koob (1999) postulated that the CRF-NA-CRF system may be essentially important in making adjustments to behaviour in response to demand. This feed-forward mechanism is particularly vulnerable to dysfunction, thus resulting in possible stress sensitisation and abnormal responses to stressors. The stress response causes an elevation in corticosterone levels. Korte and De Boer (2003) have also shown that the administration of a glucocorticoid antagonist to stressed rats increased the exploration of the open arms, indicative of a reduced anxiety state.

Mice rendered anxious by non-pharmacological means not only displayed reduction in open arm exploration but also showed reduction in general activity and rearing. The tendency to stay

in the close arms can be enhanced by compounds that increase aversion towards the anxiety provoking open arms (Korte & De Boer, 2003). Vehicle treated stressed rats spent less time in the open arms in comparison to unstressed vehicle treated rats (Korte & De Boer, 2003). In contrast anxiolytic compounds reduce the natural aversion of the open arms, treatment with 5-HT_{1A} and D₂ receptor antagonists have been shown to have anti-anxiety effects on the EPM (Rodgers & Dalvi, 1997) however results have not always been consistent with putative anxiolytic and anxiogenic compounds (Hogg, 1996).

Rodgers and Dalvi (1997) reported that prior stress increases anxiety. The findings of an EPM study conducted at our laboratory also detected anxiety 7 days after exposure to a mild situational reminder (Naciti, 2002). Other studies have reported that re-exposure to a stressor does not influence the time or number of open arm entries (Korte & De Boer, 2003; Belda *et al.*, 2004).

Lack of response to a stressor upon re-exposure may be explained as adaptation, a process in which individuals or animals display a decreased response to a repeat exposure to the same stressor (Chung *et al.*, 2000). Fear-related neural circuits are in a sustained state of hyperactivity even in the absence of a specific stressor (van Praag, 2004). Similar findings were found in victims of motor vehicle accidents that subsequently developed PTSD as compared to victims of motor vehicle accidents that did not develop PTSD (Yehuda, 1997). This adaptive mechanism can be regarded as the "behavioural shutting down" upon the recognition of the situational reminder.

The predisposition of rodents to be anxious and the interaction of this with fear induced by the EPM itself may provide researchers with more insight into the mechanisms mediating fear. Following the EPM the open field test is a popular model used to determine the absence or presence of anxiety in laboratory animals.

4.4.2 The open field test

This test has widely been used and is popular in behavioural research since it requires minimal apparatus and is quick to perform. It is based on emotionality measured by defecation and locomotor activity that takes place in a large circular arena under a high level of illumination (Lister, 1990).

Gastrointestinal activity is affected by stress and when exposed to a novel fear-provoking environment rodents tend to urinate and defecate. The number of defecation is thought to correlate emotionality and this measure is used in combination with rearing activity in the open

field test (Kask *et al.*, 2002). If motor activity were to be used as a function of anxiety, it follows that an increase in locomotion indicates decreased anxiety.

4.4.3 The hole board test

The hole board test is an apparatus that has holes in the floor through which the rodent can poke its head. This model tests the rodent's locomotor activity together with directed exploration. Head dipping assesses the exploratory behaviour of the rodent and this entity can vary independently of locomotor activity (Lister, 1990).

4.4.4 The light-dark box test

This test was originally described by Crawley and Goodwin in 1980, and is based on the natural aversion of rodents for brightly lit places. In a two compartment box, one dark and the other brightly lit, the total activity, the time spent in the light compartment and the crossing between light and dark compartments, provides information about the preference of the rodent for the dark compartment (Oliver *et al.*, 2000).

4.4.5 The social interaction test

This test is based upon the time spent by pairs of male rats novel to each other, in active social interaction, measured in a test arena (Lister, 1990). Locomotor activity assists in determining whether changes in social interaction are due to a general stimulant or sedative effect (Lister, 1990). A disadvantage of the social interaction test is that it uses pairs of animals, it is therefore difficult to examine individual behaviour (Lister, 1990).

Animal models used in the study of anxiety and stress disorders have broadened the understanding of the neuroanatomy and the neurochemistry of anxiety disorder, thus motivating the further validation of the widely used TDS model in the current study.

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Methods and Materials

Chapter 5

5.1 Introduction

This study was conducted to investigate the development and progression of severe stress-related pathology using the rodent model of Time Dependent Sensitisation (**TDS**). This already validated model (Naciti, 2002), was further investigated and characterised using certain **behavioural, biochemical and neuroendocrine** markers prior to and including **day 7 post "Re-Stress"**. This day was previously identified as the day on which the most significant changes were observed (Naciti, 2002).

All experiments were conducted on adult male Sprague-Dawley rats obtained from the Laboratory Animal Center of the Potchefstroom Campus, after approval (application number: 03D07), according to the guidelines set by the ethical committee of the North -West University.

In the current study the **Elevated Plus-Maze (EPM)**, a model used for assessing the manifestation of severe anxiety in rodents was used for studying **behavioural** changes. This model was available in our laboratory.

In order to relate behavioural outcomes to neurobiology, **biochemical parameters** were measured in the pre-frontal cortices and the hippocampi by determining concentrations of **monoamines (MA): noradrenaline, dopamine and serotonin together with their metabolites** using a validated method of **High Performance Liquid Chromatography (HPLC)** with electrochemical detection.

Corticosterone levels (CORT) in the plasma were measured as a **neuroendocrine** parameter, using a **radio immuno assay (RIA)** technique. All of the above mentioned parameters were then correlated with each other to improve insight on the disorder.

5.2 Animals

Male Sprague-Dawley rats, weighing 170-200g, provided by the Laboratory Animal Center of the Potchefstroom campus were used in all aspects of the study.

The rats were housed in cages (5 rats per cage) with a width of 28 cm, a length of 44,5 cm and a height of 12,5 cm. The conditions in the animal centre were controlled at $21 \pm 0,5^{\circ}\text{C}$ and $50 \pm 5\%$ relative humidity. Full spectrum cold white light, with a light intensity of 350–400 lux was provided over a 12 hours light – 12 hours dark cycle. A positive air pressure was maintained in the laboratory with air filtration 99,7% effective for a particle size of 2 micron and 99,9% for a particle size of 5 micron.

Food and water were readily available for consumption by the rodents.

5.3 The Time Dependent Sensitisation Model (TDS)

The behavioural model of stress-restress or **TDS** has been proposed as a useful model for PTSD (Yehuda & Antelman, 1993). Animals were stressed using a time dependent sensitisation (**TDS**) model that has been successfully used in previous studies (Harvey *et al.*, 2003).

This model involved the exposure of animals to severe stress followed by a repeat exposure to a reminder of the prior stress. The rationale being that the frequency of exposure to situational reminders contributed to the maintenance over time of fear related behavioural disturbance (Uys *et al.*, 2003).

The **TDS** model included three sequential stressors -**Triple Stressor (TS)** of escalating severity that immediately followed each other:

- 1) The rats were placed individually in a Plexiglas restrainer for two hours such that the rats were contained, but could not turn around, to limit their movement without impairing circulation to the limbs (**restraint stress**).
- 2) Immediately thereafter rats were individually placed in 18 cm of ambient water (25°C) in an upright Porsolt Apparatus for 20 minutes (**forced swim stress**).
- 3) Following that, each individual rat was immediately exposed to 0.8 ml 4% halothane vapors in a sealed container until loss of consciousness (**anesthetic inhalation stress in confined space**) whereafter the rat was placed in a Tempedair drier to dry before being returned to the cage.

The model requires rats to be left undisturbed for six days. On day 7 following the above-described **TS**, rats were "**Re-Stressed**" (**RS**) using a subtle situational reminder. This was done by the forced swim test in 18 cm of ambient water (25°C) in a Porsolt Apparatus, for 20 minutes. This subtle stressor would provide memory re-enforcement of the initial triple stressor, which then forms the basis of sensitizing the animal to the initial stressor.

According to the experimental layout (see 5.4) there were two studies:

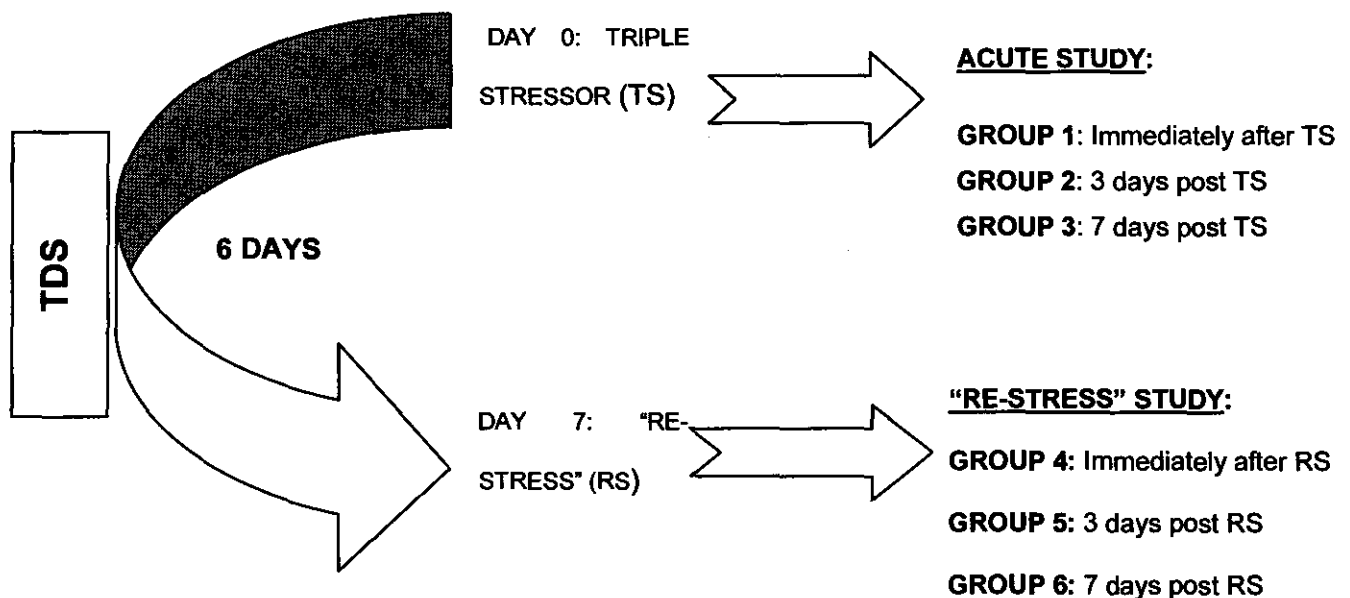
1. **The Acute Study:** Rats in Groups 1, 2 and 3 that were exposed only to the TS and
2. **The Re-Stress Study:** Rats in Groups 4, 5 and 6 were exposed to both TS and RS.

5.4 The Project aim and design

The aims of this study were to use the **biochemical, neuroendocrine and behavioural** parameters listed in 5.1 to determine:

- ❖ The difference in stress response to the TS and TS + RS thereby validating the TDS model at various points during and after the stressors in order to characterise the development and progression of stress-related pathology.

The study comprised of **six stressed experimental groups** and **one group with no induced stress as the Control group**. There were 10 rats in each of the above-mentioned groups.



Control: unstressed ($n=10$).

Figure 5.1: An outline of the study. All groups consisted of ten rats each.

In the **Acute Study** rats were exposed only to the **Triple Stressor (TS)** (see Section 5.3) and in the **Re-Stress Study** rats were left undisturbed for six days following the TS and exposed to a

subtle **situational reminder (RS)** (20 minutes of swimming in a Porsolt apparatus) on day seven. The Control group was unstressed.

Each rat, in every experimental group was evaluated on the elevated plus-maze (EPM) (refer to 5.5) (between 8:00am and 10:30am) on the day of sacrifice. Following that, the rat was sacrificed by decapitation. Trunk blood was collected into iced heparinised tubes and stored in a covered water resistant box for the period of decapitation. Blood was centrifuged and the plasma was collected and snap frozen in liquid nitrogen prior to freezing. The pre-frontal cortices and hippocampi from each rat brain were dissected on ice from the brain, placed into eppendorf tubes and then snap frozen in liquid nitrogen immediately. All decapitations occurred between 11:00am and 12:30am.

5.5 The Elevated Plus-Maze (EPM) & anxiety-like behaviour

Change in behavior that may have been caused by the TDS model was assessed using an Elevated Plus Maze (EPM). The maze consisted of two open and two enclosed arms joined at right angle to each other in the form of a "+", constructed from sturdy black Perspex. The open arms had a clear ledge of 0.5 cm on the edges to prevent the rat from falling off. The entire model was elevated about 0.6 m from the ground. Lighting conditions in the room were a single blue light bulb (40W). This model has previously been validated and used in our laboratory (Naciti, 2002).

Each rat was placed in the center of the apparatus facing the open arm, and allowed to explore the maze for 5 minutes (300 seconds) under constant surveillance of a video camera. The time was accurately monitored using a stopwatch. After each individual test the animal was returned to its cage and the apparatus wiped with methanol to eliminate odor trails.

The taped behaviour of the rat was later analysed by physically taking note of **time** (by means of a stopwatch) and counting the **number** of times each rat had entered a closed or open arm with all four paws. This raw data was noted down and then used to calculate the below mentioned ratios, which were used as an indication of anxiety.

- **Locomotion:** total number of entries was a useful index of general activity (Pellow *et al.*, 1985), which was calculated as the sum of both open and closed arm entries.

$$\text{Locomotion} = \text{sum of number of open arm entries} + \text{closed arm entries} \quad (\text{equation 5.1})$$

- **Ratio number of open arm entries:** relative to total number entries (Handley & Mathani, 1984). This ratio was used to indicate anxiety; the more anxious a rat was, the less it explored the open arms, the smaller the ratio. It was calculated as:

$$\text{Ratio number of open arm entries} = 100 \times \frac{\text{Number of open arm entries}}{\text{Total number of entries}} \quad (\text{equation 5.2}).$$

- **Ratio time in open arms:** relative to total time in arms (Pellow *et al.*, 1985) which excludes the time spent in the central square from the 300 seconds maximum time. This ratio was used to indicate anxiety, the more anxious a rat was the less time it spent in the open arms, the smaller the ratio.

$$\text{Ratio time in open arms} = 100 \times \text{Time in open arms} / \text{total time in both arms} \quad (\text{equation 5.3}).$$

Traditionally arm entries and exits have been counted when the animal crosses the threshold into an arm. An arm entry according to Lister (1987) is leaving the central square with all four paws, other researchers such as Vasar *et al.* (1993) consider an entry if only the front paws leave the central square. Automated techniques do not give data that is consistent with paw placement criteria since light beam breaks are measured (Hogg, 1996). For our observations an open arm entry was considered if the rat had all four paws in that arm.

5.6 High Performance Liquid Chromatography (HPLC) with Electrochemical Detection for biochemical correlation

Biochemical changes in the brain, that could have resulted from exposure to the TDS model, were quantified using HPLC with Electrochemical Detection (HPLC-EC). This method was proved sensitive and selective enough to detect picomolar quantities in the selected brain areas (Mori, 1991). The method of Basson (1989) was adapted for the determination of monoamine concentration in different brain areas and has been re-validated in the current study due to the purchase of new apparatus.

Electrochemical detectors measure either the conductance of the eluent or the current associated with the oxidation or reduction of solutes. When a current is passed through a solution, a reaction occurs at each electrode in which electron exchange takes place between the electrode and the substances in the solution. At the cathode, substances gain electrons (reduction) and at the anode they lose electrons (oxidation). A substance that can be oxidised or reduced is said to be electroactive (Lindsay, 1997).

Electrolysis of the sample components causes a current as a result of the electron transport. The current that passes through a working electrode (WE) is converted to a voltage reading, which is then recorded by the integrator.

5.6.1 Apparatus

Table 5.1: Experimental and technical conditions in the HPLC-analyses of monoamines and their metabolites.

Stationary phase	150 x 4.6 mm Luna C18 column (C18 reverse phase, particle size 5µm).
Detector	GBC LC 1260 EC detector
Pump	GBC LC 1120 HPLC pump
Flow Rate	1.20 ml per minute
Volume injected	50 µl loop with manual injector
Temperature (Column and room)	26 °C
Reporting integrator	Spectra-Physics (SP 4290) Integrator-chart speed 0.5 cm/min Attenuation 8.
Guard column	3.0 mm ID x 4m ml Guard Cartridge Phenomenex C18.

5.6.1.1 Mobile Phase

The mobile phase comprised of 0.1M sodium formate buffer, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium heptane sulphonic acid, 6% v/v methanol and 4% v/v acetonitrile. The pH of the buffer was set at 3.17 with the addition of 5.5 ml of orthophosphoric acid. This solution was filtered through a 0.22 µm membrane filter (Millipore) and vacuum deaerated prior to utilisation.

5.6.1.2 Calibration solution

Stock solutions of the biogenic amines and their acid metabolites were made in the same 0.1 M perchloric acid solution (10.87 ml/l of distilled water) (containing 0.5 mM sodium metabisulphite (0.09505 g/l) and 0.3 mM Na₂EDTA (0.1117 g/l). The solution was kept refrigerated and

aliquots of these solutions were mixed to yield stock standard solutions that were used to calibrate the integrator on a daily basis.

5.6.2 Monoamine standards

5.6.2.1 Preparation of internal standard- isoprenaline

N-isopropyl-DL-Noradrenaline-hydrochloride=247.72 MW (Aldrich)

5 mg was weighed and dissolved in 50 ml of calibration solution. 30 μ l of this solution was taken and made up to 2 ml with 0.1M perchloric acid (see 5.6.1.2). The final concentration was 1500 ng/ ml. Isoprenaline was used as internal standard throughout all determinations.

Stock solutions of the monoamines were prepared using each of the components that were to be analysed. The following steps give the specifications of mass of each component used in stock solution 1. Stock solution 2 was prepared from stock solution 1.

5.6.2.2 Preparation of Noradrenaline- NA standard

L-Noradrenaline hydrochloride- $C_8H_{11}NO_3 \cdot HCl$ = 205.6407 MW (Fluka)

$Na-C_8H_{11}NO_3$ = 169.1798 MW (82.27%)

6.08 mg was weighed and dissolved in 2 ml of 0.1 M perchloric acid solution (see 5.6.1.2), 82.27% of 6.08 mg was then 5 mg, which represented the sodium portion of the total compound.

5.6.2.3 Preparation of 3,4-Dihydroxyphenylacetic acid-DOPAC

DOPAC=168.15 MW (Aldrich)

5 mg was weighed and dissolved in 2 ml of 0.1 M perchloric acid (see 5.6.1.2).

5.6.2.4 5-Hydroxyindole-3-acetic acid –5HIAA

5-HIAA=191.19 MW (Aldrich)

5 mg was weighed and dissolved in 2 ml of 0.1 M perchloric acid (see 5.6.1.2).

5.6.2.5 Dopamine – DA

3-Hydroxytyramine hydrochloride β - (3,4-Dihydroxyphenyl)-aethylamine hydrochloride=189.64 MW (Aldrich)

5 mg was weighed and dissolved in 2 ml of 0.1 M perchloric acid (see 5.6.1.2).

5.6.2.6 Homovanillic acid- HVA

HVA=182.18 MW (Aldrich)

5 mg was weighed and dissolved in 2 ml of 0.1 M perchloric acid (see 5.6.1.2).

5.6.2.7 Serotonin – 5HT

Serotonin creatinine sulphate (5-Hydroxytryptamine creatinine sulphate)

$C_{14}H_{21}N_5O_6S \cdot H_2O$ = 405.43 MW (Merck)

Serotonin= 176.2 MW (43.46%).

11.504 mg was weighed and dissolved in 2 ml of 0.1 M perchloric acid (see 5.6.1.2), 43.46% of the 11.504 mg represented 5 mg of the serotonin part of the total compound.

Stock solution 2 was prepared by diluting 40 μ l of **stock solution 1** with 1960 μ l of 0.1 M perchloric acid (see 5.6.1.2), to yield a concentration of 10 μ g/ml.

5.6.3 Sample preparation

Following dissection of the pre-frontal cortex and the hippocampus from the rat brain, these areas were placed individually into polypropylene tubes and immediately snap frozen in liquid nitrogen (-198°C).

On the day of analysis, samples were weighed and thawed; 1 ml of a 0.1 M perchloric acid solution (see 5.6.1.2) was added to each tube. The tissue in each tube was then ruptured by sonication (2 x 12 seconds, at an amplitude of 14 μ), which is the preferred method of cell disruption for small sample and volume size (Keller *et al.*, 1976).

The tubes were then allowed to stand on ice for 20 minutes to complete perchlorate precipitation of proteins and extraction of monoamines. Following this period, samples were centrifuged at 4°C in a Sorvall Discovery ultra centrifuge (Model 9056) manufactured by Hitachi at 16 000 revolutions per minute (24 000 x g).

The supernatant was removed, transferred to a clean polypropylene tube and immediately frozen in liquid nitrogen. Prior to HPLC analyses samples were thawed.

5.6.4 Sample injection

Each sample of frozen tissue extract was thawed just prior to injection into the HPLC column. The pH of the solution was adjusted to 5 with the addition of 1 μ l of 10 M potassium acetate. The potassium acetate was prepared by dissolving 98.04 g of potassium acetate (Merck) in 100 ml of distilled water.

An aliquot of 200 μ l of the tissue extract was removed with a pipette and placed into another eppendorf tube with an aliquot of 20 μ l of isoprenaline as internal standard (IS). The final solution was vortexed and 50 μ l was manually injected into the HPLC column.

5.6.5 Calculation of concentrations

On the day on which the samples were injected, two standard solutions of the monoamine and metabolite mixture (25 ng/ml and 200 ng/ml) were prepared from the mother solution. The 25 ng/ml standard solution was prepared by diluting 5 μ l of stock solution 2 with 1995 μ l of 0.1M perchloric acid solution. The 200 ng/ml standard solution was prepared by diluting 40 μ l of stock solution 2 with 1960 μ l of 0.1M perchloric acid solution.

From each of the standard solutions 200 μ l was pipetted into an amber eppendorf tube and 20 μ l of isoprenaline was added as the internal standard. 50 μ l of these samples were injected into the HPLC and allowed to run until the last compound (serotonin) in the sample had eluted (30 minutes). The concentrations of the components were then calculated as AUC of standard divided by AUC of isoprenaline. The integrator was calibrated daily with these two solutions prior to analysis to account for inter-day variations in experimental conditions.

The experimental samples each yielded a chromatogram. Monoamine content was determined by dividing the area under the curve (AUC) of each of the monoamines in the sample by the AUC of IS in the sample. This ratio was then converted by means of a calibration curve of the AUC of the standard solution. The average concentration was divided by the mass of the concerned brain area. Results were expressed as ng/g wet weight of tissue. Metabolite turnover ratios were determined using the equation: Metabolite/Monoamine (Altar *et al.*, 1986).

5.6.6 Validation of method

The described method was validated in terms of its selectivity, linearity, range and repeatability.

5.6.6.1 Specificity and selectivity

Specificity and selectivity refers to the ability of an analytical method to accurately analyse a component in the presence of other components such as biological materials and other metabolic products.

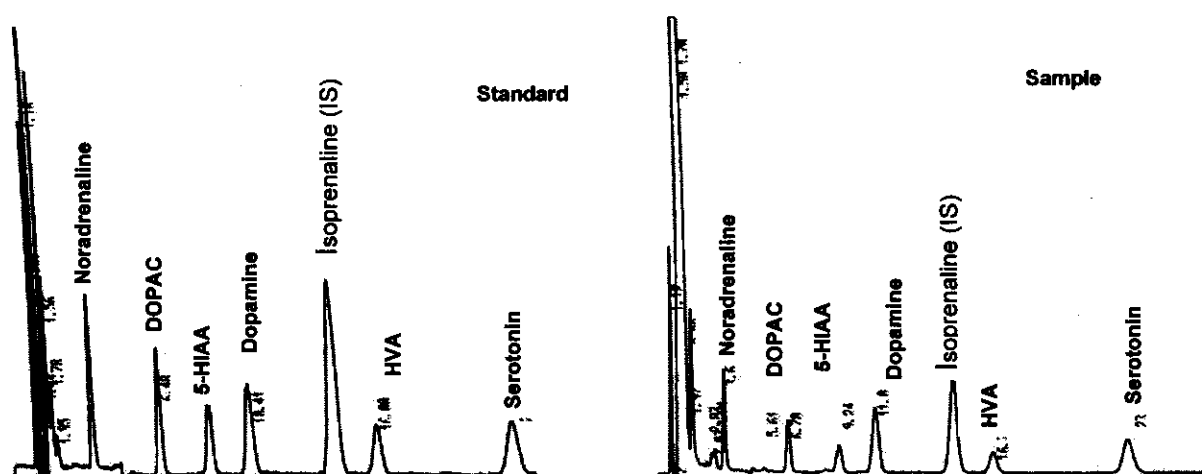


Figure 5.2: HPLC chromatographs of a standard solution and brain sample.

This method proved sensitive and selective since there is no overlap of compounds in both the standard solution and the sample.

5.6.6.2 Linearity

Good linearity between the peak-surface areas over the concentration limits is used in drawing up standard curves. The peak surface area ratio is plotted against concentration. The best straight line through these points is drawn. The equation:

$$y = mx + c$$

Where y = peak surface area ratio; m = gradient; x = concentration in ng/ ml; c = y intercept was used and the values are depicted in Table 5.2.

Table 5.2: The gradient, y-intercept and regression values of the standard curves.

Monoamine/ metabolite	Gradient (m)	y-intercept (c)	Regression (r²)
Noradrenaline	820.81	12266	0.9969
DOPAC	938.05	12893	0.9987
5-HIAA	765.38	12478	0.9948
Dopamine	1399.3	16807	0.9982
HVA	777.37	24724	0.9928
Serotonin	1484.4	17918	0.9966

5.6.6.3 Range

This method proved sensitive enough to detect a minimum of 10 ng/ ml and the maximum limit detected was 200 ng/ ml of the standard solutions.

5.6.6.4 Repeatability

The repeatability was determined by injecting three concentrations of every sample that was to be analysed, into the HPLC consecutively. This was done by injecting three solutions of different concentrations six consecutive times. The surface area ratio was calculated using the area under the curve (AUC) of the standard divided by the area under the curve of the internal standard.

$$\text{Surface area ratio} = \text{AUC standard} / \text{AUC internal standard} \quad (\text{equation 5.4})$$

The % RSD was calculated according to **equation 5.5**.

$$\% \text{ RSD} = \text{Standard error variation (STDEV)} / \text{Mean AUC ratio} \quad (\text{equation 5.5})$$

For a method to be repeatable according to USP (2002) specifications the average % RSD must be smaller than 10%.

Table 5.3: The average % RSD of the monoamines and their metabolites

Monoamine / Metabolite	Average % RSD
Noradrenaline	5.822253
DOPAC	5.148422
5-HIAA	7.115169
Dopamine	5.918645
HVA	5.268425
Serotonin	5.460373

5.7 Plasma corticosterone concentration as neuroendocrine correlate

Corticosterone concentrations were determined as neuroendocrine indication of stress. The initial idea was to develop a HPLC method with ultra-violet (UV) detection for the measurement of corticosterone levels in the plasma. The method was validated in water standards but could not be validated in plasma. In retrospect reasons cited for this are: a failure to purchase activated charcoal for the removal of endogenous corticosterone, an unsuitable column and the degradation of the sample when samples were dried in a water-bath. Due to time constraints corticosterone levels were then determined using a radio immunoassay (RIA) kit.

Corticosterone levels of all the rats used in this study were determined using a Coat-A-Count Rat Corticosterone kit (TKRC1). This kit was commercially available from Diagnostic Products Corporation (DPC). The 100-tube kit contains less than 4.5 micro curies of radioactive ^{125}I rat corticosterone.

It is a solid-phase radio immunoassay in which ^{125}I -labeled rat corticosterone competed for 2 hours with corticosterone in the sample for antibody sites. Because the antibody was immobilized to the wall of a polypropylene tube, decanting the supernatant was sufficient to terminate the competition and isolate the radiolabeled corticosterone. Counting the tube in a gamma counter then yielded a number, which was converted by way of a calibration curve to measure the corticosterone present in the sample.

5.7.1 Sample collection

Trunk blood was collected into iced heparinized tubes immediately after decapitation. All blood samples were kept on ice for the period in which sacrifice took place. Since corticosterone is influenced by a circadian rhythm all samples were collected at the same time of day (between 11am and 12:30am).

The collected blood samples were then centrifuged at 5000 x g for 10 minutes at 4 °C to remove cells from the plasma. 2 ml of plasma was removed and collected into a 2.5 ml amber colored eppendorf tube using a pipette. These tubes were immediately snap frozen and stored at -198 °C until subsequent determinations. The samples were thawed at room temperature before analysis.

5.7.2 Radio immunoassay (RIA) Procedure

- The kit contained 100 amber colored tubes, 105 ml iodinated rat corticosterone ¹²⁵I in liquid form and eight vials of rat corticosterone calibrators prepared in corticosterone free rat serum.
- All components of the kit were at room temperature (15-28 °C) before use.
- Two transparent polypropylene tubes (12 x 75 mm) were labeled for the total counts (T). Into these two tubes 1 ml of ¹²⁵I Rat Corticosterone was pipetted. These tubes were vortexed and covered with parafilm. Both tubes were not decanted and were placed directly into the gamma counter after the two-hour incubation period.
- Sixteen of the amber colored Rat Corticosterone Ab-Coated tubes that were included in the kit was labeled alphabetically from A to H in duplicate.
- The remainder of the tubes was labeled according to experimental group name and number of sample, in duplicate.
- All tubes were placed in upright foam decanting racks.
- 50 µL of each of the calibrators listed below were pipetted into the tubes A to H.

Table 5.4: Calibrator concentration used for the construction of the RIA standard curve

Calibrator	Nanogram/ ml
A	0
B	20
C	50
D	100
E	200
F	500
G	1000
H	2000

- Serum was directly assayed. 50 μ L of each serum sample was pipetted using a 50 μ L automatic pipette into the marked two tubes.
- Into each tube 1 ml of ^{125} I Rat Corticosterone was added using an automated 1000 μ L pipette. This was done within 10 minutes.
- Each tube was vortexed and left to stand for 2 hours at room temperature.
- When two hours had lapsed, the liquid in the tubes was emptied into a radioactive waste container.
- After decanting thoroughly, all visible traces of moisture were removed by striking the tubes on absorbent sheets.
- The tubes were drained for an additional 2 minutes on absorbent paper and all visible traces of moisture were removed.
- The tubes were loaded in the gamma-counter racks. They were placed in the sequence in which they were read by the gamma counter (Cobra-Auto gamma, Packard).
- The transparent polypropylene tubes (with its contents) were loaded first and read as the Total Counts.

- ❑ Tube A to H was loaded in chronological order. The average reading of each concentration was used for the construction of a standard curve.
- ❑ Each tube from the experimental samples was then counted for one minute by the gamma counter (Cobra-Auto gamma, Packard).
- ❑ The results were read by interpolation from the standard curve.
- ❑ Results (expressed as ng/ ml) were printed out.

5.8 Statistical analysis

The Statistical Consultation Services of the North-West University conducted all statistical analyses of the results.

All data was first analysed using a one-way analysis of variance (ANOVA) (StatSoft, Inc.2001: Statistica Data Analysis Software System, version 6). If significant differences were noted using the ANOVA, multiple comparisons were performed using the **Dunnett's t-test** to compare all experimental groups to the control group with statistical significance defined at 5% (* $p < 0.05$) level. In addition the **Bonferroni-corrected 5% t-test** was performed on a 98.27% level of confidence ($p < 0.0173$) i.e. one third of 5%, to confirm any statistically significant difference between the three groups of the Acute Study and the three groups of the Re-Stress Study. All data are expressed as the mean $\pm$ Standard Error of the Mean (SEM). The p value is an indication of the probability to reject the null-hypothesis which states that the groups being compared are similar.

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Experimental Results

Chapter 6

6.1 Introduction

The TDS model used in this study has previously been validated in Sprague-Dawley rats at certain time intervals following the Re-Stress. **Day 7 post Re-Stress** was identified as the day on which the most profound changes of statistical significance were noticed (Naciti, 2002). In this study the TDS model was further validated using *behavioural, biochemical and neuroendocrine* observations noticed on specific days prior to and including the above-mentioned day.

The effects of the TDS model was analysed using the **Elevated Plus-Maze (EPM)** as a model that confirmed **behavioural** changes. Changes in **Monoamine (MA)** concentration of the hippocampus and the pre-frontal cortex were determined as **biochemical** correlates and **plasma corticosterone (CORT)** concentrations were determined as a **neuroendocrine** observation.

The study lay-out consisted of two sub-studies: a) An **Acute Study** to determine the efficacy of the **Triple Stressor** in inducing **stress** and b) a **Re-Stress Study** to determine the difference in response to the TS and RS, thereby further validating the TDS model at various time points during and post stressors in order to characterise the development and progression of stress-related pathology. Each group comprised of ten rats.

The **Acute Study** consisted of three groups of Sprague-Dawley rats that had been exposed to the **Triple Stressor**, tested on the EPM prior to decapitation following which brain tissue and blood was collected to determine MA and CORT concentrations as follows:

- **Group 1:** immediately following the Triple Stressor (TS stat)
- **Group 2:** 3 days following the Triple Stressor (3 days TS)
- **Group 3:** 7 days following the Triple Stressor (7 days TS)

Another three groups were used in the **Re-Stress Study**. These Sprague-Dawley rats were exposed to the **Triple Stressor**, left undisturbed for 6 days and then exposed to the **situational reminder on day 7** where after the above mentioned parameters were also measured:

- **Group 4:** immediately following the Re-Stress (RS stat),
- **Group 5:** 3 days post Re-Stress (3 days RS),
- **Group 6:** 7 days post Re-Stress (7 days RS).

An additional group, **Control** was included as a unstressed control Group.

In comparison to **Control**, statistical significant results using the Dunnett's t-test are indicated as ^{*}p value which is depicted on the graphs as ^{*}. Statistical differences observed when inter-group comparisons were conducted within the three groups of the Acute and Re-stress Study using the Bonferroni-corrected 5% t-test are indicated by a p-value with a superscript e.g. [#]p shown on the graph as #.

6.2 Behaviour Study: Elevated Plus Maze (EPM)

The EPM uses aversive behaviour as an indication of anxiety. The maze consisted of two open arms, two closed arms and a central square. The more anxious a rat is the less likely it is to explore and spend time in the open arms. Anxiety was measured using ratio **number of open arm entries**, ratio **time in open arms** and **locomotion** as indicators (see 5.5). All data was obtained after the video taped behaviour of the rat had been manually analysed.

6.2.1 Ratio number of open arm entries

An entry was recognised if all four paws of the rat were in a specific arm. The ratio number of open arm entries is calculated using **equation 5.2** by counting the number of times the rat crosses over into an open arm with all four paws. This value is then divided by the total number of entries and expressed as a percentage. A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups [$F(6,58) = 2.53$, $p < 0.0305$]. Data was further analysed by the Dunnett's t-test to confirm significant differences between each of the groups and the control ($p < 0.05$).

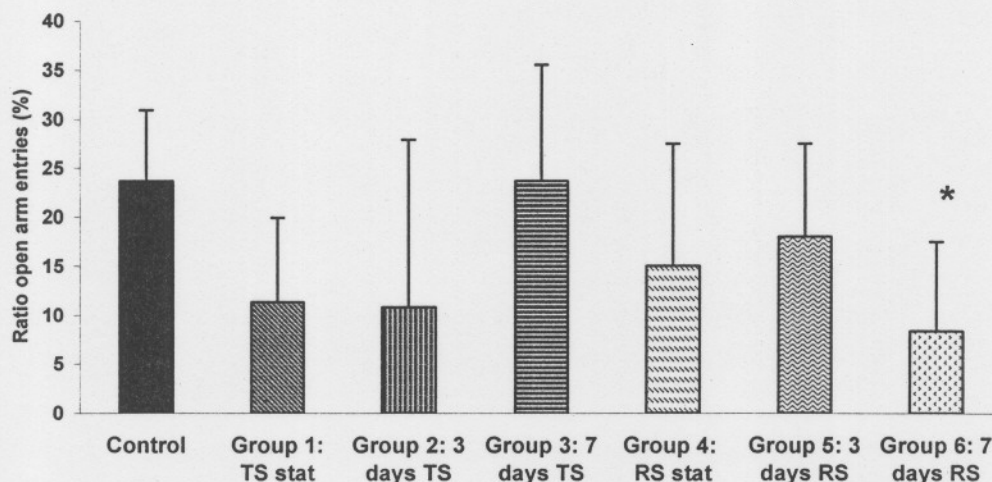


Figure 6.1: Ratio number of open arm entries observed on different days following the Triple Stressor and Re-Stress (mean $\pm$ SEM; $n=10$), * $p<0.05$ vs. Control (Dunnett's t-test).

In the **Acute Study**, the Triple Stressor had no statistically significant effect on the ratio open arm entries as compared to Control in Groups 1,2 and 3 although a tendency towards increased anxiety was evident. Inter-group comparisons using the **Bonferroni-corrected 5% t-test** also showed no significant differences between these groups.

In the **Re-Stress Study**, the ratio open arm entries were studied on three different days (immediately, 3 days and 7 days) following exposure to the situational reminder. Data analysed using a **Dunnett's t-test** showed a significant decrease in the ratio open arm entries in Group 6 (7 days post RS) as compared to Control ($p<0.05$). This finding indicated that the rats in Group 6 were more anxious than the Control. Although Group 4 and 5 appeared to have decreased ratio open arm entries, inter-group comparison using the **Bonferroni-corrected 5% t-test** showed no significant differences between any of the groups in the **Re-Stress Study**.

6.2.2 Ratio time in open arms

This ratio was calculated using **equation 5.3** by dividing the time each rat spent in the open arm (measured in seconds) by the total time spent in both arms (calculated as the sum of the time the rat spent in the open and closed arm with all four paws). It was also used as an indication of anxiety. A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups [$F(6,56) = 3.25$, $p<0.0023$]. Data was then analysed using the Dunnett's t-test to confirm significant differences

between each of the groups and the Control ($p < 0.05$). Subsequently the **Bonferroni-corrected 5% t-test** ($p < 0.0173$) was performed to note inter-group differences.

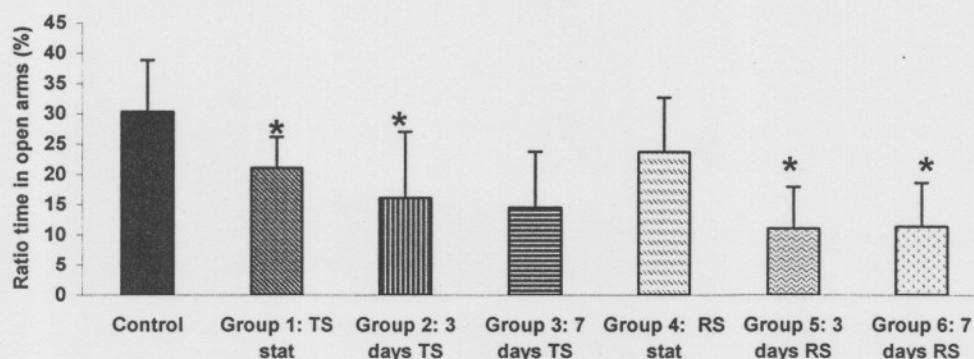


Figure 6.2: The ratio time in spent in open arms (%) observed on different days following the Triple Stressor and Re-Stress, (mean $\pm$ SEM ($n=10$)) * $p < 0.05$ vs. Control (Dunnett's t-test).

The effect of the Triple Stressor on the ratio time spent in open arms was studied in the **Acute Study**. Group 1 (immediately following the Triple Stressor) and Group 2 (3 days following the Triple Stressor) showed a statistically significant decrease in the ratio time spent in open arms in comparison to the Control ($p < 0.05$; **Dunnett's t-test**) indicating anxiety. It appeared that Group 3 (7 days post TS) also had a decreased ratio time in open arms, but this was not supported as a statistically significant difference. Inter-group comparison using the **Bonferroni-corrected 5% t-test** showed no significant differences between these groups.

In the **Re-stress Study**, according to **Figure 6.2**, Group 5 (3 days post RS) and Group 6 (7 days post RS) showed a statistically significant decrease ($p < 0.05$; **Dunnett's t-test**) in ratio time spent in open arms confirming that both groups were more anxious than the Control. Group 4 (immediately after RS) showed no statistically significant difference in the ratio time spent in open arms. Inter-group comparison using the **Bonferroni-corrected 5% t-test** also showed no significant differences between these groups.

6.2.3 Locomotion

Locomotion was calculated as the sum of the number of entries the rat made into both arms (equation 5.1). The more anxious a rat is the less activity it displays in the arms. A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control

showed no significant differences across the groups [$F(6,61) = 1.59$, $p < 0.1663$]. Data was then analysed using the Dunnett's *t*-test to confirm significant differences between each of the groups and the Control ($p < 0.05$). Subsequently the Bonferroni-corrected 5% *t*-test ($p < 0.0173$) was performed to note inter-group differences.

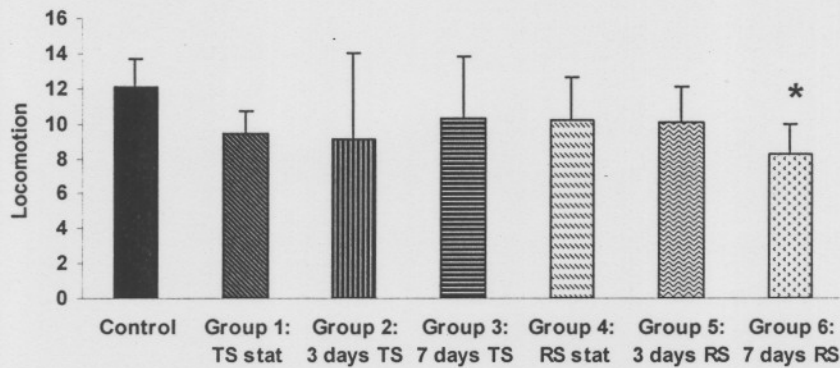


Figure 6.3: Locomotion observed on different days following the Triple Stressor and Re-Stress (mean ± SEM ($n=10$), * $p < 0.05$ vs. Control (Dunnett's *t*-test)).

In the **Acute Study**, the effect of the TS on locomotion showed no significant differences between these groups using both the Dunnett's *t*-test and the Bonferroni-corrected 5% *t*-test.

The **Re-stress Study** (Figure 6.3) showed Group 6 (7 days post RS) to have a statistically significant decrease in locomotion as compared to Control indicative of anxiety ($p < 0.05$). Inter-group comparison using the Bonferroni-corrected 5% *t*-test showed no significant differences between these groups.

6.3 Plasma Corticosterone Analysis

Plasma corticosterone concentrations were measured using a radio immuno assay (RIA) kit. This determination was included as a **neuroendocrine** correlate of stress. A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups [$F(6,63) = 19.51$, $p < 0.0001$]. Data was then analysed using the Dunnett's *t*-test to confirm significant differences between each of the groups and the Control ($p < 0.05$). Subsequently the Bonferroni-corrected 5% *t*-test ($p < 0.0173$) was performed to note inter-group differences.

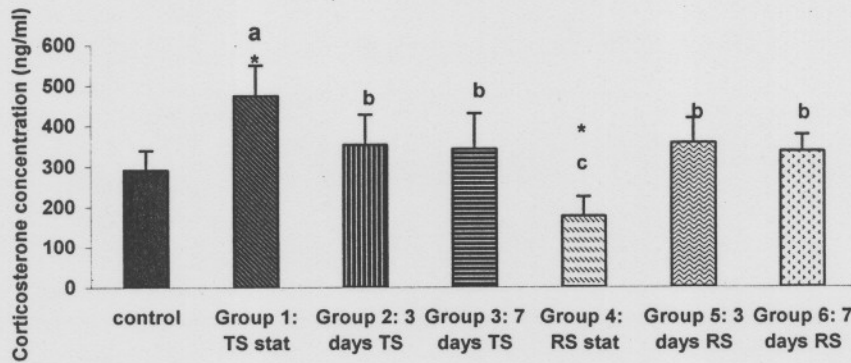


Figure 6.4: Plasma corticosterone concentrations observed on different days following the Triple Stressor and Re-Stress (mean $\pm$ SEM; $n=10$); * $p<0.05$ vs. Control (Dunnett's t-test); ^{a, b} $p<0.0173$ vs. Group 1-6 (Bonferroni-corrected 5% t-test).

According to **Figure 6.4**, in the **Acute Study**, **Dunnett's t-test** showed a statistically significant increase in plasma corticosterone concentrations in Group 1 (immediately following the Triple Stressor) as compared to the Control. This indicated that stress was induced in these rats.

The effect was not maintained at a constant level through-out the Acute Study. The **Bonferroni-corrected 5% t-test** recognised a statistically significant decrease in plasma corticosterone concentrations between Group 1 (immediately after TS) as compared to Group 2 (3 days post TS) and Group 3 (7 days post TS). No statistically significant difference was noted between Groups 2 and 3.

In the **Re-Stress Study**, Group 4 (immediately after Re-Stress), showed a statistically significant decrease ($p<0.05$; **Dunnett's t-test**) in plasma corticosterone concentration as compared to the Control. The **Bonferroni-corrected 5% t-test** confirmed Groups 5 (3 days post RS) and 6 (7 days post RS) to have statistically significant increased corticosterone concentrations as compared to Group 4 (immediately after Re-Stress), but found no differences between groups 5 and 6. The Re-Stress study showed statistically significant decreases in corticosterone levels in Group 5 and 6 in relation to Group 1.

6.4 Monoamine concentrations in the pre-frontal cortex

6.4.1 Noradrenaline

A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups [$F(6,56) = 5.78$, $p<0.0001$]. Data was then analysed using the Dunnett's t-test to confirm significant differences between each of

the groups and the Control ($p < 0.05$). Subsequently the **Bonferroni-corrected 5% t-test** ($p < 0.0173$) was performed to note inter-group differences.

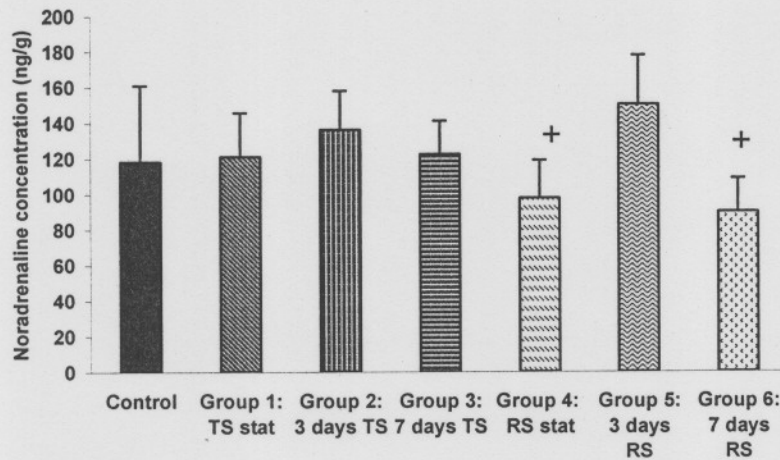


Figure 6.5: The mean $\pm$ SEM ($n=9$) values of noradrenaline concentrations in the PFC observed on different days following the Triple Stressor and Re-Stress * $p < 0.0173$ vs. Group 5 (Bonferroni-corrected 5% t-test).

In the **Acute Study** no significant differences across the groups were noted using both the **Dunnett's t-test** and the **Bonferroni-corrected 5% t-test**.

The **Re-Stress Study** also indicated no significant differences across the groups using the **Dunnett's t-test** however, the **Bonferroni-corrected 5% t-test** according to **Figure 6.5**, showed statistically significant lower NA concentrations in both Groups 4 (immediately after RS) and 6 (7 days post RS) compared to Group 5 (3 days post RS).

6.4.2 Dopamine

A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups [$F(6,57) = 7.76$, $p < 0.0001$]. Data was then analysed using the **Dunnett's t-test** to confirm significant differences between each of the groups and the Control ($p < 0.05$). Subsequently the **Bonferroni-corrected 5% t-test** ($p < 0.0173$) was performed to note inter-group differences.

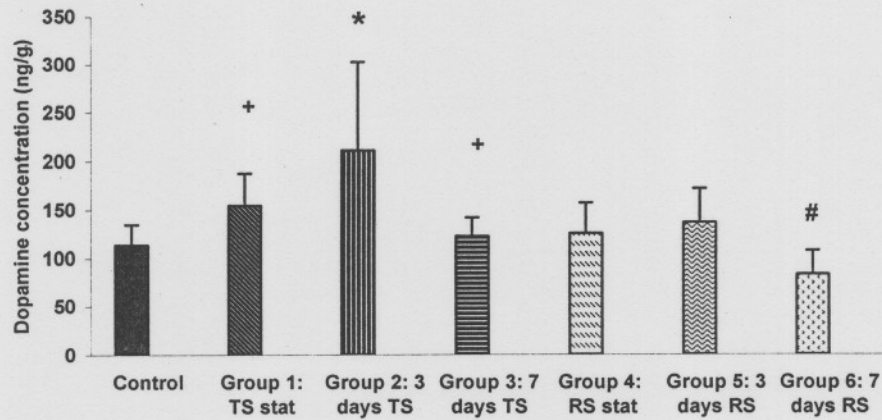


Figure 6.6: The mean $\pm$ SEM ($n=10$) values of Dopamine concentrations in the PFC observed on different days following the Triple Stressor and Re- Stress (* $p < 0.05$ vs. Control (**Dunnett's t-test**); + $p < 0.0173$ vs. Group 2; # $p < 0.0173$ vs. Group 5 (**Bonferroni-corrected 5% t-test**)).

According to **Figure 6.6** in the **Acute Study**, Group 2 (3 days after Triple Stressor), showed a statistically significant increase ($p < 0.05$; **Dunnett's t-test**) in DA concentration as compared to the Control. Inter-group comparison using the **Bonferroni-corrected 5% t-test** confirmed this to be a significant increase in relation to both Group 1 (immediately after Triple Stressor) and Group 3 (7 days post Triple Stressor).

In the **Re-Stress Study**, data analysed using the Dunnett's t-test showed no significant differences in comparison to Control. Inter-group comparison using the **Bonferroni-corrected 5% t-test** showed Group 6 (7 days post RS) to have decreased concentration of DA in relation to Group 5 (3 days post RS) ($p < 0.0173$).

6.4.3 Serotonin

A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed no significant differences across the groups [$F(6,60) = 1.16$ $p > 0.3379$]. Data was then analysed using the **Dunnett's t-test** and subsequently the **Bonferroni-corrected 5% t-test** to confirm no significant differences.

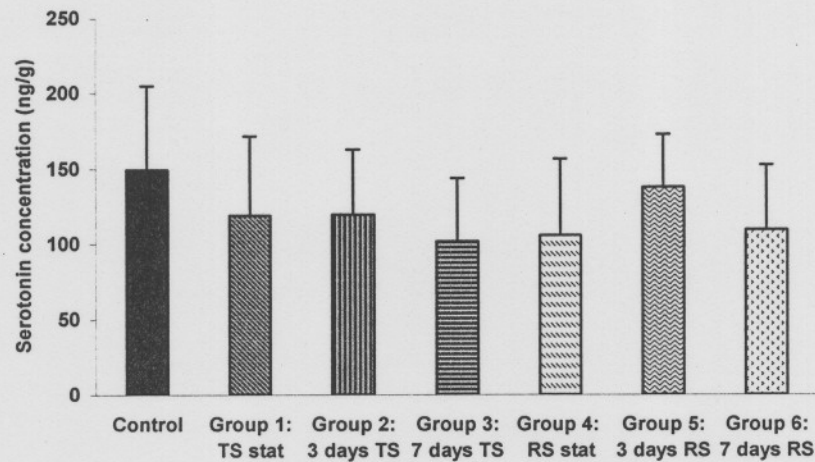


Figure 6.7: The mean $\pm$ SEM ($n=10$) values of Serotonin concentrations in the PFC observed on different days following the Triple Stressor and Re-Stress.

According to **Figure 6.7** no statistically significant differences were noted between the groups of both the **Acute and Re-stress Studies**, even though there appeared to be a tendency towards decreased concentration.

6.4.4 Monoamine metabolites

A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups. DOPAC [$F(6,41) = 3.54$, $p < 0.0057$], 5-HIAA [$F(6,40) = 17.73$, $p < 0.0001$] and HVA [$F(4,24) = 6.64$, $p < 0.0010$]. Data was then analysed using the Dunnett's t -test to confirm significant differences between each of the groups and the Control ($p < 0.05$). Subsequently the **Bonferroni-corrected 5% t -test** ($p < 0.0173$) was performed to note inter-group differences. If no value has been assigned to a column, this indicates that even though the metabolite was detected it was not quantified in all samples.

Table 6.1: The mean $\pm$ SEM ($n=10$ unless indicated otherwise) values of monoamine metabolites in the PFC observed on different days following the Triple Stressor and Re-Stressor (* $p < 0.05$ vs. Control (Dunnett's t-test); $^{\dagger}p < 0.0173$ vs. Group 1; $^{*Z}p < 0.0173$ vs. Group 2; $^ap < 0.0173$ vs. Group 5 (Bonferroni-corrected 5% t-test).

	DOPAC (ng/g)	HVA (ng/g)	5-HIAA (ng/g)
Control	13.807 $\pm$ 9.24 $n=4$	131.672 $\pm$ 62.39 $n=3$	38.619 $\pm$ 25.32 $n=7$
Group 1: TS stat	38.362 $\pm$ 13.82 *	38.924 $\pm$ 20.35 * $n=7$	25.099 $\pm$ 19.10 ^{Z} $n=5$
Group 2: 3 days TS	38.018 $\pm$ 16.39 $n=7$	47.854 $\pm$ 1.63 * $n=2$	86.1955 $\pm$ 50.60 $n=6$
Group 3: 7 days TS	17.740 $\pm$ 3.09 *† $n=9$	21.366 $\pm$ 4.42 * $n=9$	20.992 $\pm$ 12.42 ^{Z}
Group 4: RS stat	36.799 $\pm$ 20.49	55.209 $\pm$ 22.64 * $n=8$	40.283 $\pm$ 39.86 ^{a} $n=9$
Group 5: 3 days RS	37.690 $\pm$ 19.99 $n=8$	-	290.412 $\pm$ 16.54 * $n=2$
Group 6: 7 days RS	20.771 $\pm$ 8.510 $n=5$	-	55.5247 $\pm$ 51.15 ^{a} $n=8$

6.4.4.1 DOPAC

According to Table 6.1 **DOPAC concentrations** were statistically significantly elevated in Group 1 using the Dunnett's t-test ($p < 0.05$) as compared to Control. In the Acute Study Group 3 was found to have a statistically significant reduction in DOPAC concentrations when compared to Groups 2 and 1 ($p < 0.0173$) using the Bonferroni-corrected 5% t-test.

In the Re-Stress Study, inter-group comparisons using the Bonferroni-corrected 5% t-test showed no statistically significant differences across the groups.

6.4.4.2 HVA

HVA concentrations were found significantly decreased in comparison to Control in all of the groups in which it was quantified i.e. Groups 1,2,3,4, using **Dunnett's t-test** ($p < 0.05$).

6.4.4.3 5-HIAA

5-HIAA concentrations in the **Acute Study** showed Group 2 to have a statistically significant increase in concentration as compared to Control ($p < 0.05$; **Dunnett's t-test**). The elevated concentration noted in Group 2 also differs from both Groups 1 and 3 ($p < 0.0173$; **Bonferroni-corrected 5% t-test**).

The **Re-Stress Study** indicates that 5-HIAA concentrations are elevated in Group 5 as compared to Control ($p < 0.05$; **Dunnett's t-test**). Inter-group comparison using the **Bonferroni-corrected 5% t-test** confirmed the elevation as significant ($p < 0.0173$) in relation to Groups 4 and 6.

6.4.5 DOPAC/DA and 5-HIAA/5-HT ratios

Metabolite ratios were calculated by dividing the metabolite i.e. DOPAC or 5-HIAA by its monoamine i.e. DA or 5-HT (see 5.6.4). Since HVA was not detected in all experimental groups this metabolite was not used in calculating dopamine turnover. A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed no significant differences across the groups for DOPAC/DA [$F(6,43) = 2.11$, $p > 0.07201$] but 5-HIAA/5-HT indicated differences [$F(6,39) = 3.63$, $p < 0.0059$]. Data was then analysed using the Dunnett's t-test to confirm significant differences between each of the groups and the Control ($p < 0.05$). Subsequently the **Bonferroni-corrected 5% t-test** ($p < 0.0173$) was performed to note inter-group differences.

Table 6.2: The mean $\pm$ SEM values of monoamine turnover in the PFC observed on different days following the Triple Stressor and Re-Stressor (* $p < 0.05$ vs. Control (Dunnett's t-test); $^+p < 0.0173$ vs. Group 5 (Bonferroni-corrected 5% t-test)).

	DOPAC/DA	5-HIAA/5-HT
Control	0.121	0.258
Group 1: TS stat	0.248	0.211
Group 2: 3 days TS	0.180	0.720
Group 3: 7 days TS	0.145	0.206
Group 4: RS stat	0.294	0.458 ⁺
Group 5: 3 days RS	0.277	2.117*
Group 6: 7 days RS	0.251	0.510 ⁺

DOPAC/DA ratio in the PFC was not statistically different in any of the experimental groups as compared to the Control.

5-HIAA/5-HT in the PFC showed increased concentrations in Group 5 as compared to the Control ($p < 0.05$; Dunnett's t-test), this increase was significant as compared to Group 4 and Group 6 ($p < 0.0173$; Bonferroni-corrected 5% t-test).

6.5 Monoamine concentrations in the Hippocampus

Monoamines and their metabolites in the hippocampus were determined using HPLC with electrochemical detection.

6.5.1 Noradrenaline

A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups [$F(6,60) = 55.31$, $p < 0.0001$]. Data was then analysed using the Dunnett's t-test to confirm significant differences between each of the groups and the Control ($p < 0.05$). Subsequently the Bonferroni-corrected 5% t-test ($p < 0.0173$) was performed to note inter-group differences.

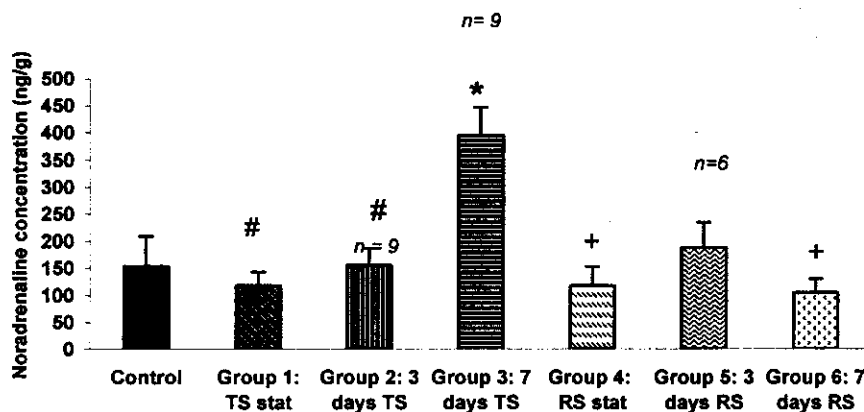


Figure 6.8: The mean $\pm$ SEM ($n=10$ unless stated otherwise) values of Noradrenaline concentration in the hippocampus observed on different days following the Triple Stressor and Re-Stressor ($^{\#}p < 0.05$ vs. Control (Dunnett's t-test); $^{\#}p < 0.0173$ vs. Group 3, $^*p < 0.0173$ vs. Group 5 (Bonferroni-corrected 5% t-test).

In the **Acute Study** Group 3 (7 days post TS) showed a statistically significant increase ($p < 0.05$; **Dunnett's t-test**) in noradrenaline concentrations when compared to the Control. Inter-group comparisons confirmed both Group 1 (immediately after TS) and Group 2 (3 days post TS) to be statistically significantly lower than Group 3 (7 days post TS) ($p < 0.0173$; **Bonferroni-corrected 5% t-test**)

In the **Re-Stress Study** the **Bonferroni-corrected 5% t-test** ($p < 0.0173$) confirmed both Groups 4 (immediately after RS) and Group 6 (7 days post RS) to be of decreased concentration as compared to Group 5 (3 days post RS).

6.5.2 Dopamine

A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups [$F(6,61) = 26.66$, $p < 0.0001$]. Data

was then analysed using the Dunnett's t-test to confirm significant differences between each of the groups and the Control ($p<0.05$). Subsequently the Bonferroni-corrected 5% t-test ($p<0.0173$) was performed to note inter-group differences.

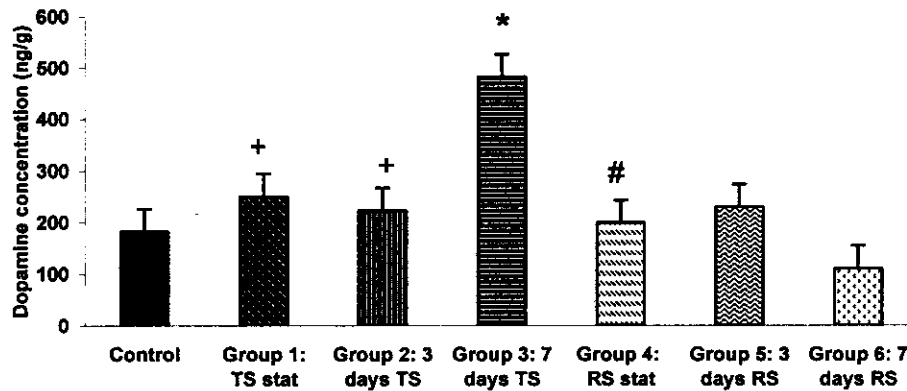


Figure 6.9: The mean $\pm$ SEM ($n=10$) values of Dopamine concentration in the hippocampus observed on different days following the Triple Stressor and Re-Stressor (* $p<0.05$ vs. Control (Dunnett's t-test); # $p<0.0173$ vs. Group 6 ; + $p<0.0173$ vs. Group 3 (Bonferroni-corrected 5% t-test)).

In the **Acute Study** dopamine concentrations in the hippocampus were found elevated in Group 3 (7 days post TS) as compared to the Control ($p<0.05$; **Dunnett's t-test**). Inter-group comparisons analysed using the **Bonferroni-corrected 5% t-test** also confirmed Group 3 (7 days post TS) to be statistically significantly elevated ($p<0.0173$) as compared to Group 1 (immediately after TS) and Group 3 (3 days post TS).

The **Re-Stress Study** showed no statistically significant difference using the **Dunnett's t-test**, however inter-group comparisons using the **Bonferroni-corrected 5% t-test** showed Group 6 (7 days post RS), to be statistically lower ($p<0.0173$) than Group 4 (immediately after RS) in concentration.

6.5.3 Serotonin

A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups [$F(6,56) = 19.59$, $p<0.0001$]. Data was then analysed using the **Dunnett's t-test** to confirm significant differences between each of the groups and the Control ($p<0.05$). Subsequently the **Bonferroni-corrected 5% t-test** ($p<0.0173$) was performed to note inter-group differences.

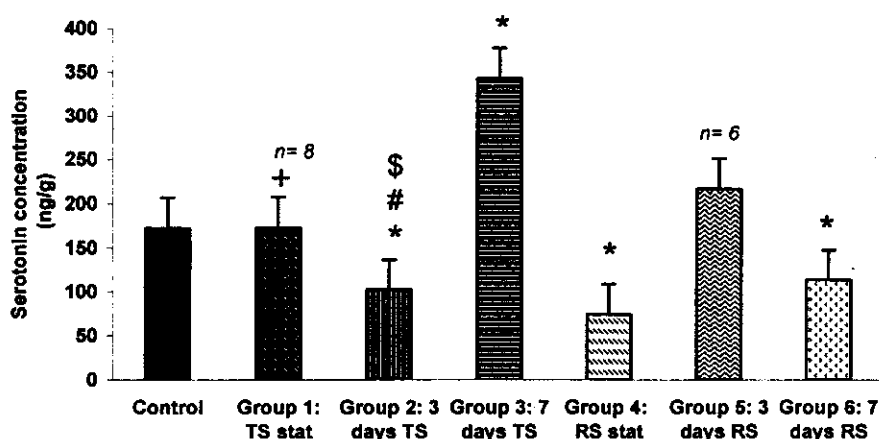


Figure 6.10: The mean $\pm$ SEM ($n=10$) values of serotonin concentration in the hippocampus observed on different days following the Triple Stressor and Re-Stressor (* $p<0.05$ vs. Control (Dunnett's t-test); * $p<0.0173$ vs. Group 3; \$ $p<0.0173$ vs. Group 1 (Bonferroni-corrected 5% t-test)).

In the **Acute Study** Group 2 (3 days post TS) was shown to have decreased ($p<0.05$; **Dunnett's t-test**) concentration in comparison to Control. Group 3 (7 days post TS) exhibited elevated serotonin concentration when compared to the Control ($p<0.05$; **Dunnett's t-test**). Group 1 (immediately after TS) had a higher concentration ($p<0.0173$; **Bonferroni-corrected 5% t-test**) than Group 2 (3 days post TS). Group 3 (7 days post TS) had a statistically significant increased ($p<0.0173$; **Bonferroni-corrected 5% t-test**) concentration in comparison to both Group 1 (immediately after TS) and Group 2 (3 days post TS).

6.5.4 Monoamine metabolites

A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups. DOPAC [$F(5,32) = 95.03$, $p<0.0001$], 5-HIAA [$F(6,23) = 53.72$, $p<0.0001$]. Data was then analysed using the Dunnett's t-test to confirm significant differences between each of the groups and the Control ($p<0.05$). Subsequently the **Bonferroni-corrected 5% t-test** ($p<0.0173$) was performed to note inter-group differences.

Table 6.3: The mean $\pm$ SEM ($n=10$ unless stated otherwise) values of monoamine metabolites in the hippocampus observed on different days following the Triple Stressor and Re-Stressor. (* $p<0.05$ vs. Control (**Dunnett's t-test**); $^{\dagger}p<0.0173$ vs. Group 1; $^{\#}p<0.0173$ vs. Group 3; $^ap<0.0173$ vs. Group 3; $^bp<0.0173$ vs. Group 4 (**Bonferroni-corrected 5% t-test**)).

	DOPAC (ng/g)	HVA (ng/g)	5-HIAA (ng/g)
Control	1.904 $\pm$ 9.27 $n=9$	-	33.600 $\pm$ 23.50 $n=4$
Group 1: TS stat	25.380 $\pm$ 13.82 † $n=4$	-	53.516 $\pm$ 52.18 ^{a} $n=4$
Group 2: 3 days TS	20.360 $\pm$ 16.40 $^{\dagger\#}$ $n=7$	-	23.433 $\pm$ 1.74 ^{a} $n=3$
Group 3: 7 days TS	310.392 $\pm$ 3.01 *	-	253.552 $\pm$ 49.47 * $n=9$
Group 4: RS stat	15.351 $\pm$ 20.50 $n=7$	243.411 $\pm$ 46.67	186.616 $\pm$ 01 * $n=1$
Group 5: 3 days RS	-	98.421 $\pm$ 85.514	-
Group 6: 7 days RS	4.564 $\pm$ 8.52 $n=6$	-	18.672 $\pm$ 27.58 ^{b} $n=9$

6.5.4.1 DOPAC

In the **Acute Study** Group 3 (7 days post TS) showed a significant elevation when compared to the Control ($p<0.05$; **Dunnett's t-test**). Inter-group comparisons using the **Bonferroni-corrected 5% t-test** confirmed Group 1 (immediately after TS) and Group 2 (3 days post TS) to be of a lower concentration ($p<0.0173$) in comparison to Group 3 (7 days post TS).

In the **Re-Stress Study**, this metabolite was not detected in Group 5 (3 days post RS). The **Dunnett's t-test** showed Group 4 (3 days post RS) to be significantly elevated ($p<0.05$) as compared to the Control.

6.5.4.2 HVA

HVA was not quantified in the Control and in other experimental groups. Even though it was detected in Group 4 and 5, this metabolite was not used in characterisation of the TDS model.

6.5.4.3 5-HIAA

In the **Acute Study** Group 3 (7 days post TS) showed a significant elevation when compared to the Control ($p<0.05$; **Dunnett's t-test**). Inter-group comparisons using the **Bonferroni-**

corrected 5% t-test confirmed Group 1 (immediately after TS) and Group 2 (3 days post TS) to be of a lower ($p<0.0173$) concentration than Group 3 (7 days post TS).

In the **Re-Stress Study**, this metabolite was not detected in Group 5 (3 days post RS). The **Dunnett's t-test** showed Group 4 (3 days post RS) significantly elevated ($p<0.05$) as compared to the Control. The **Bonferroni-corrected 5% t-test** indicated Group 4 (immediately after RS) to be of a higher ($p<0.0173$) concentration than Group 6 (7 days post RS).

6.5.5 Metabolite ratios

Metabolite turnover was calculated by dividing the metabolite i.e. DOPAC/ 5-HIAA by its monoamine i.e. DA/ 5-HT. Since HVA was not detected in all experimental groups this metabolite was not used in calculating dopamine turnover. A one-way analysis of variance (ANOVA) performed to compare all experimental groups and the control showed significant differences across the groups DOPAC/DA [$F(5,30) = 29.28$, $p<0.0001$] & 5-HIAA/5-HT [$F(6,22) = 37.27$, $p<0.0001$]. Data was then analysed using the Dunnett's t-test to confirm significant differences between each of the groups and the Control ($p<0.05$). Subsequently the **Bonferroni-corrected 5% t-test** ($p<0.0173$) was performed to note inter-group differences.

Table 6.4: The mean $\pm$ SEM values of monoamine turnover in the hippocampus observed on different days following the Triple Stressor and Re-Stressor (* $p < 0.05$ vs. Control (**Dunnett's t-test**); $^+p < 0.0173$ vs. Group 3; $^*p < 0.0173$ vs. Group 4; $^{\$}p < 0.0173$ vs. Group 3 (**Bonferroni-corrected 5% t-test**)).

	DOPAC/DA	5-HIAA/5-HT
Control	0.010	0.177
Group 1: TS stat	0.101 ⁺	0.346 ^{\$}
Group 2: 3 days TS	0.091 ⁺	0.230 ^{\$}
Group 3: 7 days TS	0.645 [*]	0.740 [*]
Group 4: RS stat	0.077	2.525 [*]
Group 5: 3 days RS	-	-
Group 6: 7 days RS	0.040	0.165 [#]

Data analysed using the **Dunnett's t-test** showed significant differences across the groups. Inter-group comparison using the **Bonferroni-corrected 5% t-test** confirmed significant differences between these groups at a 98.267% level ($p < 0.0173$).

The DOPAC/DA ratio in the hippocampus was increased in experimental Group 3 (7 days post TS) as compared to the Control ($p < 0.05$; **Dunnett's t-test**). Groups 1 and 2 in the **Acute Study** are statistically decreased as compared to Group 3 ($p < 0.0173$; **Bonferroni-corrected 5% t-test**).

5-HIAA/5-HT ratio in the hippocampus showed statistically significant elevation ($p < 0.05$; **Dunnett's t-test**) between Group 3 (7 days post TS) and the Control. In the **Acute Study** Groups 1 and 2 showed a statistically significant lower 5-HIAA/5-HT ratio as compared to Group

3 ($p < 0.0173$; **Bonferroni-corrected 5% t-test**). The implications of all results will be explained in the ensuing chapter.

6.6 Summary of statistically significant changes

Only statistically significant results obtained from experimental groups as compared to control ($p < 0.05$; **Dunnett's t-test**) are depicted in **Table 6.5**. Inter-group comparisons that were conducted using the **Bonferroni-corrected 5% t-test** ($p < 0.0173$) have been excluded from the results reported in **Table 6.5**.

Table 6.5: Summary of statistically significant changes found in the entire study as compared to control ($p < 0.05$; Dunnett's t-test). *None* indicates no change of significance.

	ACUTE STUDY			RE-STRESS STUDY		
	Group 1: TS stat	Group 2: 3 days TS	Group 3: 7 days TS	Group 4: RS stat	Group 5: 3 days RS	Group 6: 7 days RS
<u>Behavioral anxiety</u>	increase	increase	none	none	increase	increase
<u>corticosterone</u>	increase	none	none	decrease	none	none
<u>DA: PFC</u>	none	increase	none	none	none	none
<u>DA: Hippocampus</u>	none	none	increase	none	none	none
<u>DOPAC: PFC</u>	increase	none	none	none	none	none
<u>DOPAC: Hippocampus</u>	none	increase	increase	none	none	none
<u>DOPAC/DA: PFC</u>	none	none	none	none	none	none
<u>DOPAC/DA: Hippocampus</u>	none	none	increase	none	none	none
<u>HVA: PFC</u>	decrease	decrease	decrease	decrease	none	none
<u>HVA: Hippocampus</u>	none	none	none	none	none	none
<u>5-HT: PFC</u>	none	none	none	none	none	none
<u>5-HT: Hippocampus</u>	none	decrease	increase	decrease	none	decrease
<u>5-HIAA: PFC</u>	none	none	none	none	increase	none
<u>5-HIAA: Hippocampus</u>	none	none	increase	increase	none	none
<u>5-HIAA/5-HT: PFC</u>	none	none	none	none	increase	none
<u>5-HIAA/5-HT: Hippocampus</u>	none	none	increase	increase	none	none
<u>NA: PFC</u>	none	none	none	none	none	none
<u>NA: Hippocampus</u>	none	none	increase	none	none	none

6.7 References

NACITI, C. 2002. Pharmacological and behavioral assessment of an animal model of PTSD. MSc dissertation, Potchefstroom University for CHE

Discussion

Chapter 7

7.1 Introduction

The Diagnostic and Statistical Manual of Psychiatric Disorders (DSM-IV) classifies post traumatic stress disorder (PTSD) as an anxiety disorder, comprising of three symptom clusters: **re-experiencing** the event, with the resultant symptoms of numbness and **avoidance**, and **hyper arousal** (American Psychiatric Association, 1994). Dysfunction in the pre-frontal cortex and the hippocampus are specifically implicated in the neuroendocrine and cognitive dysfunctions that are observed in PTSD.

This study aimed to characterize the changes that occur in brain monoamine concentration in these regions from the initial exposure to trauma (Acute Study) until 7 days following exposure to a situational reminder (Re-Stress Study) and to correlate these changes, if any, to anxiety-like behavior and plasma corticosterone concentrations.

7.2 Findings of the Acute Study

This section discusses the neuroendocrine and behavioural changes that occur following the Triple Stressor (TS). These changes were examined on three days: on the day of exposure to the TS (TS stat), 3 days post TS and 7 days post TS.

7.2.1 Corticosterone concentration

In response to the Triple Stressor (Group 1), **Figure 6.4 & Table 6.5**, a statistically significant increase in plasma corticosterone concentration as compared to the basal values of the unstressed control was found.

This was consistent with other findings that have indicated that exposure to an acutely stressful event leads to the rapid release of corticosterone (Kanter *et al.*, 2001; Oosthuizen, 2003). Increased corticosterone levels are necessary for the establishment of homeostasis and consolidation of the information associated with the event in order to maximise the likelihood of survival in the case of a re-encounter (Korte, 2001).

The decreased hippocampal volume observed in patients with PTSD has in part been ascribed to increased exposure of the hippocampus to glucocorticoids (Sapolsky, 2000). It is possible

that the massive secretion of corticosterone upon exposure to the trauma is the first step in a cascade of events leading to a maladaptive HPA-axis response leading to hippocampal atrophy and subsequent memory deficits of PTSD.

Although the hippocampus regulates corticosterone release through its inhibitory actions on the HPA-axis (Bremner, 1999), hippocampal damage would result in the disruption of the negative feedback loop, thereby making the hippocampus increasingly vulnerable to the effects of glucocorticoid toxicity (Nutt, 2000). Pitman (1989) hypothesised that PTSD is a product of exposure to an extremely traumatic event, resulting in an exaggerated response of neuropeptides and inappropriate memory formation, thus maintaining an organism in a state of heightened arousal.

In this study, on day 3 following exposure to the TS (Group 2), corticosterone concentrations displayed a marked decrease from the elevated concentration observed immediately after exposure to the TS. The CORT levels in this group were not statistically different in relation to the Control (**Figure 6.4**).

Analyses of plasma samples on 7 days post TS (Group 3), revealed that the corticosterone concentration was not statistically different in comparison to the Control and Group 2 (3 days post TS), thereby implicating that corticosterone levels are stabilised at a level that does not differ from basal suggesting a gradual shutting down of the stress response during the 7 day period.

7.2.2 Behavioural analyses

The significant increase in corticosterone in response to the TS, is also supported by the display of anxiety-like behaviour noted in Group 1 (TS stat). According to **Table 6.5 & Figure 6.2**, statistically significant anxiety was detected on the EPM following exposure to the TS. Korte and De Boer (2003) have also shown that the administration of a glucocorticoid antagonist to stressed rats increased the exploration of the open arms, indicative of the role of increased glucocorticoids in the genesis of anxiety.

Marked anxiety was also detected on day 3 post TS (Group 2) (**Table 6.5**) as depicted in **Figure 6.1** and **Figure 6.2**. The observed anxiety in both Group 1 and Group 2 is consistent with findings of other studies that have also reported a decreased exploration of the open arms following exposure to a severe stressor (Hogg, 1996; Korte & De Boer, 2003).

Group 3 (7 days post TS) showed no statistically significant behavioural change in any of the determinants used as an indication of anxiety. This implies that the anxiety-like behaviour

manifested immediately after exposure to the Triple Stressor became gradually lost over 7 days. In contrast, exposure to predator stress has been shown to maintain an enhanced anxiety state for as long as 3 weeks post exposure (Korte & De Boer, 2003).

7.2.3 Serotonin, 5-HIAA and 5-HIAA/5-HT ratio in the PFC and hippocampus

The majority of preclinical models of anxiety suggest that increased serotonergic function is associated with anxiety i.e. increased serotonin results in increased aversive behaviour.

In the current study, serotonin, 5-HIAA and the 5-HIAA/5-HT ratio in the **PFC** displayed no statistically significant alteration, although a tendency in the direction of decreased concentration seemed apparent (**Figure 6.7**). Increased corticosterone levels, as noted in the Acute Study are known to selectively increase 5-HT_{2A} receptor activity in the PFC (Katagiri *et al.*, 2001) and this receptor has been implicated in playing a role in anxiety.

Activation of the 5-HT_{2A} receptors further activates the HPA-axis (Heimrick-Leucke & Evans, 2002) and stimulates the release of ACTH and corticosterone. Pre-clinical data suggests that the antagonism of the 5-HT_{2A} receptor decreases both the behavioural and physiological response to stress (Kent *et al.*, 2002).

Activation of the 5-HT_{2A} receptors in the PFC impedes adaptation, a process in which individuals or animals display a decreased response to a repeat exposure to the same stressor (Chung *et al.*, 2000). Previous studies conducted in our laboratory using the same TDS model have confirmed an increase in 5-HT₂ receptor affinity in the PFC (Harvey *et al.*, 2003). This may account for the lack of adaptation following a stressful event. No change of statistical significance in PFC 5-HIAA (**Table 6.1**) and the ratio of 5-HIAA/ 5-HT (**Table 6.2**) was observed in the Acute Study. Muira and co-workers (2002) also reported unchanged 5-HIAA levels in response to a novel stressor.

Hippocampal serotonin concentrations in response to the TS were found dysregulated in comparison to the Control (**Figure 6.10**). In response to the TS, no statistically significant change in hippocampal 5-HT was observed immediately after the Ts in relation to Control. A microdialysis study conducted by Kirby *et al.* (1997) has also reported no change in hippocampal serotonin concentration following 100 minutes of restraint stress. Interestingly a decrease was noted on day 3 post TS and an increase was noted on day 7 post TS, both of which were statistically significant.

Even though the immediate response to the TS showed no statistically significant change in 5-HT concentrations, restraint stress has been shown to induce suppression of 5-HT_{1A} receptor binding and atrophy of the dendrites of the CA3 hippocampal pyramidal neurons (Watanabe *et al.*, 1992), while Harvey and co-workers (2003) have found that TDS stress increases 5-HT_{1A} receptor affinity.

5-HT_{1A} receptors exert an inhibitory effect on the hippocampus and increased corticosterone levels as were found in response to the TS, decreased this inhibitory effect (Montgomery *et al.*, 2001). Due to the co-localisation of glucocorticoid receptors in the hippocampus, altered corticosterone levels are known to influence serotonergic activity (Montgomery *et al.*, 2001).

As mentioned earlier, on day 3 post TS, the **hippocampal** serotonin concentration (**Figure 6.10 & Table 6.5**) as compared to the Control, showed a decrease of significance in comparison to both Groups 1 (TS stat) and 3 (7 days post TS). Low serotonin in rats is characterised by hyperarousal (van der Kolk, 1994), thus explaining the presence of anxiety-like behaviour noted on the EPM.

In the ensuing days it is interesting that the statistically significant decrease in hippocampal 5-HT levels observed on day 3 post TS, has been increased to a level of statistical significance by day 7 post TS (Group 3) as compared to Control (**Figure 6.10 & Table 6.5**). Increased serotonergic activity in the CNS is required for the successful adaptation to stress and the prevention of subsequent learned helplessness (Spivak *et al.*, 1999). Hippocampal 5-HIAA (**Table 6.3**) and the ratio of 5-HIAA/ 5-HT (**Table 6.4**) also increased to a level of significance by day 7 post TS.

Removal of corticosterone by adrenalectomy results in an anatomically specific decrease in 5-HT metabolism, while stressful procedures increase serotonin turnover (Nishi & Azmitia, 1996).

7.2.4 Noradrenaline concentrations in the PFC and hippocampus

Analyses of noradrenaline concentrations in both the **PFC (Figure 6.5)** and the **hippocampus (Figure 6.8)** revealed no significant alteration. Since the LC also indirectly stimulates the HPA-axis via the limbic system (De Bellis *et al.*, 1999), an increased NA concentration following exposure to the TS was expected. Results of a micro-dialysis study conducted by Shimizu and co-workers (1994) have reported immobilisation stress to increase NA levels by 12.8% as compared to basal NA levels in the PFC.

When a normal rise in CORT is prevented by an adrenalectomy, acute stress results in an even greater increase in plasma NA levels in rodents (Brown & Fisher, 1986). The normal rise in CORT levels following a stressor, as has been observed in this study, has also been noted in human studies. CORT release is accompanied by an immediate release of NA followed by a transient decrease in NA levels (Laakman *et al.*, 1984) in blood plasma. Increased NA concentrations have been noted in the urine (De Bellis *et al.*, 1999).

Stress has also been shown to produce regionally selective increases in noradrenaline turnover in the limbic region of the brain (Charney *et al.*, 2000). In PTSD, there is a failure to shut-down the stress response as evident from the raised NA levels. This implies that a perpetual stress response is maintained. A delayed, but significant increase in **hippocampal** noradrenaline concentrations were found on day 3 post TS (Table 6.5).

Elevated NA levels have been associated with the laying down of emotional memories (Carrasco, 1997) and enhanced consolidation of emotion laden aversive events (van Praag, 2004). Activation of the noradrenergic system plays an important integrative function in coping and adaptation to stress (Koob, 1999). Elevated NA are however known to impair PFC function resulting in deeply engraved traumatic memories that are expressed as flashbacks, nightmares and intrusive recollections (Pitman, 1989). This study indicated no change of statistical significance in the PFC concentrations of NA (Figure 6.5).

7.2.5 Dopamine, HVA & DOPAC and the DOPAC/DA ratio in the PFC and hippocampus

Changes in dopamine transmission particularly in the PFC, occur in response to threatening challenges (Reis *et al.*, 2004). There is evidence of the involvement of dopamine in aversive states elicited by stressful stimuli. Behavioural studies aimed at disclosing the role of dopamine in anxiety have reported anxiolytic-like, anxiogenic-like and lack of effects with the use of dopamine agonists and antagonists in animal models of anxiety (Rodgers *et al.*, 1994).

Stress has been shown to have varying effects on the DA system of the PFC. Dopaminergic innervation of the PFC is highly sensitive to stress. Exposure to an inescapable stressor such as the TS used in this study, has been found to selectively suppress the activation of the DA system of the PFC (Berridge, 2002), but increased PFC dopamine concentrations in response to stress have also been reported (Pani *et al.*, 2000). In the current study, no statistically significant change in the DA of the PFC was found in response to the TS.

However, DA levels appear to increase in the ensuing days to reach a level of significance by day 3 post TS. Detch and co-workers (1990) also reported increased dopamine concentrations in the PFC following acute stress.

Excessive PFC dopaminergic activity, as has been noted, has a negative effect on cognitive function, resulting in inappropriate selecting and processing of environmental stimuli. Optimal cognitive functioning thus depends on an optimal range of dopamine turnover (Pani *et al.*, 2000).

The elevated DA concentration noted in the PFC on day 3 post TS was not maintained and DA levels drop back to basal levels by day 7 post TS. This stabilisation may indicate a successful adaptation response. Sensitisation of the dopaminergic system of the PFC has been paralleled to kindling, and may be relevant to the pathogenesis of PTSD (Bonne *et al.*, 2003).

In the PFC, concentrations of DOPAC show much variation indicative of both an accelerated and slower rate of dopamine metabolism in comparison to Control (Table 6.5). The main metabolite of DA, HVA however was found decreased in comparison to the Control group.

Hippocampal dopamine concentrations were unchanged after exposure to the TS until day 3 post TS as compared to the Control. It is interesting that statistically increased hippocampal DOPAC concentrations were observed on day 3 post TS (Group 2) as compared to Control (Table 6.5). This increased DOPAC is found to be consistent with findings in the literature that indicate that mild stress increases dopaminergic activity as a coping attempt made by the animal (Gamaro *et al.*, 2003).

By day 7 post TS, Group 3 indicates a significantly elevated dopamine concentration in the **hippocampus**. After stress, an increase in DA concentration occurs which suppresses limbic DA transmission, thereby protecting against positive psychotic symptoms (Pani *et al.*, 2000). Increased DA concentrations have been linked most directly with intrusive symptoms of PTSD (Yehuda *et al.*, 1992).

Another indicator of heightened dopaminergic activity in the hippocampus was the elevated ratio of DOPAC/DA (Table 6.5). This elevation in the DOPAC/DA ratio was significant as compared to the other experimental groups in the Acute Study. This increase may be attributed to CRF since administration of exogenous CRF resulted in an increased DA concentration leading to the hypothesis that endogenous CRF is involved in the increase of dopaminergic activity (Murphy *et al.*, 2003).

Activation of the D₂ receptors by DA occurs in response to an innate fear stimulus while a combined activation of both D₂ and D₁ receptors seem to be involved in the acquisition of a conditioned avoidance response (Reis *et al.*, 2004). Thus dopamine through the actions of these two receptors may strengthen the aversiveness triggered by a conditioned response (Reis *et al.*, 2004).

Severe stress does not suppress dopamine- β -hydroxylase activity (Hamner *et al.*, 1998), thus psychosis in PTSD might involve noradrenergic instead of dopaminergic hyperactivity (Hamner & Gold, 1998). Dopamine- β -hydroxylase activity may modify the phenomenology of PTSD by facilitating the experience of psychotic symptoms but further work is needed to make a conclusive deduction (Hamner & Gold, 1998).

7.2.6 Summary: Acute Study

The results of this study indicate that the TS did induce stress in rodents as evident from the elevated **corticosterone** concentrations and **behavioural anxiety**. The frontal cortex in humans, primates and rats is a large functionally heterogeneous region, thought to be important for the orchestration of a number of separate cognitive processes including working memory components and suppression of previous behavioural strategies. This executive function is believed to facilitate the matching of an appropriate behavioural strategy to rapid changes in task requirements (Broad *et al.*, 2002).

This study did not exhibit distinct changes in **PFC MA** levels with the exception of decreased HVA concentrations and elevated DA concentrations observed on day 3 post TS. The **hippocampal MA** and metabolite concentrations showed more profound dysregulation. The anxiety detected in Group 2 can be correlated to a hippocampal serotonergic deficit.

The delayed elevation in NA levels observed in Group 3 (7 days post TS) highlights dysregulation of the noradrenergic system accounting for the deficits in a variety of cognitive and affective disorders that are in turn associated with PTSD. Central noradrenergic as well as dopaminergic systems possess a robust compensatory mechanism that permits long-term adjustment in activity (Berridge & Waterhouse, 2003).

Hippocampal MA levels are significantly increased in relation to Control on day 7 post TS. Interestingly on day 7 post exposure to the TS, anxiety-like behaviour was absent and corticosterone concentrations had stabilised on a level insignificantly different from basal levels. This implies that behaviourally the rats seem to have recovered after exposure to the TS, but on a biochemical level dysregulation was prominent.

7.3 Findings of the Re-Stress Study

This section explains the response that was observed at three different time points following exposure to the RS i.e. RS stat, 3 days post RS, 7 days post RS.

7.3.1 Corticosterone concentration

An important vulnerability factor for the induction of PTSD is exposure to a prior trauma. Thus exposure to a situation only remotely similar to the initial trauma, such as the forced swim in the case of the TDS model results in a highly emotional response (Bonne *et al.*, 2003).

PTSD differs from other anxiety disorders in that it can be associated with below basal corticosterone levels (Kanter *et al.*, 2001), as was found in this particular study (**Figure 6.4**). Low levels of cortisol impair memory functions that are essential for an effective stress response (Sapolsky, 2000), thereby impairing proper appreciation of the actual situation making the differentiation of relevant and irrelevant information difficult.

Decreased cortisol levels can be ascribed to a hypersensitive negative feedback mechanism and a sensitisation of the inhibitory elements of the HPA-axis (Liberzon *et al.*, 1997). Behavioural abnormality has accordingly been attributed to the under stimulation of MRs (van Praag, 2004).

Findings of previous studies concerning corticosterone changes observed in PTSD have not been consistent. Human studies conducted by De Bellis and co-workers (1999) reported increased cortisol levels in patients diagnosed with PTSD, in contrast some animal studies have reported a peripheral hypocortisolism (Harvey *et al.*, 2003; Kanter *et al.*, 2001). There are also findings that do not support the concept of either a static "hypo/hyper-cortisolism" in PTSD but rather suggest a psychogenic basis for cortisol alterations in PTSD in relation to psychosocial stress. This indicates of a central regulatory HPA-axis dysfunction characterised by a dynamic tendency to overreact in both upward and downward directions (Mason *et al.*, 2002). It is interesting that the display of bi-directional expression of symptoms, a typical quality of PTSD, is an important quality of the TDS model (Uys *et al.*, 2003).

By day 3 post RS (Group 5) corticosterone concentrations were not found to be statistically different from the Control. the decreased level that was found after exposure was therefore not maintained at the below basal concentration. **Figure 6.4** indicates corticosterone concentrations to be slightly elevated in comparison to the basal level, although this is not regarded as statistically significant.

On day 7 post RS, corticosterone concentrations showed no statistically significant alteration in comparison to Control and Group 5. This observation is contrary to the findings of others using the TDS model (Harvey *et al.*, 2003), where decreased concentrations were noted on day 7 post RS. The corticosterone levels, however differed significantly from the elevated level noted after the TS (Group 1) and from the below basal level found after exposure to the RS (Group 4).

This dynamic tendency to overreact in both upward and downward directions in response to a stressor and in the absence of a stressor to exhibit stability suggest a psychogenic basis for cortisol alterations in PTSD (Mason *et al.*, 2002). As eluded to previously this finding highlights an advantage of the TDS model that allows bi-directional expression of symptoms (Yehuda & Antelman, 1993; Uys *et al.*, 2003). Thus central regulatory HPA-axis dysfunction ascribed to a hypersensitive negative feedback mechanism and a sensitisation of the inhibitory elements of the HPA-axis (Liberzon *et al.*, 1997) may be present.

Although highly speculative, exposure to a third situational reminder could result in corticosterone levels even lower than that observed in Group 4 (immediately after exposure to situational reminder). This speculation is based upon the findings of a previous study conducted at our laboratory (Oosthuizen, 2003) that has confirmed corticosterone levels to be significantly reduced on day 21 post RS. This would suggest that repeated trauma, through either flashbacks, re-experiencing or subtle environmental cues reminiscent of the original trauma, will propagate illness severity over time, a hallmark trait of PTSD.

7.3.2 Behavioural analyses

According to Table 6.5 (Figures 6.1, 6.2 & 6.3), no anxiety-like behaviour was detected immediately after exposure to the situational reminder (RS) compared to the Control. This may be explained as adaptation, a process in which individuals or animals display a decreased response to a repeat exposure to the same stressor (Chung *et al.*, 2000).

Fear-related neural circuits are in a sustained state of hyperactivity even in the absence of a specific stressor (van Praag, 2004). Similar findings were found in victims of motor vehicle accidents that subsequently developed PTSD as compared to victims of motor vehicle accidents that did not develop PTSD (Yehuda, 1997). This adaptive mechanism can be regarded as the "behavioural shutting down" upon the recognition of the situational reminder.

Similar results were obtained from a study based on the same principle as the TDS model. Prior exposure to a stressor followed by a "re-stress" did not affect the time or number of open arm entries (Belda *et al.* 2004). Partial immersion in water for 15 minutes, similar to the 20 minutes

swim stress of the situational reminder, also did not influence % time in open arms (Korte & De Boer, 2003).

Group 5 (3 days post RS) displayed behavioural anxiety (Table 6.5). A statistically significant decrease in the ratio time spent in open arms (Figure 6.2) as compared to the control was noted. This finding is consistent with the report of Rodgers and Dalvi (1997) that found prior stress exposure to increase anxiety. Mice rendered anxious by non-pharmacological means not only displayed reduction in open arm exploration but also showed reduction in general activity and rearing. By day 7 post RS, rats were extremely anxious. The anxiety detected in Group 6 (7 days post RS) showed a statistically significant decrease (Table 6.5) in all of the determinants of anxiety (Figures 6.1, 6.2 and 6.3) as compared to Control. This finding is consistent with the results of a previous EPM study conducted at our laboratory (Naciti, 2002).

7.3.3 Serotonin, 5-HIAA and 5-HIAA/5-HT ratio in the PFC and hippocampus

According to Figure 6.7 no statistically significant differences in 5-HT concentrations in the PFC were observed in the Re-Stress Study. According to Table 6.5, 5-HIAA concentrations in the PFC were found elevated on day 3 post RS. This elevation was also found to be statistically significant in comparison to Group 4 (immediately after RS) and Group 6 (7 days post RS) (Table 6.1). Decreased cerebrospinal 5-HIAA concentrations have been reported to positively correlate with anxiety since 5-HIAA is to a large extent a function of serotonin metabolism (van Praag, 2004 a). Souza and De Loon (1986) have found an inverse relationship between plasma corticosterone concentrations and 5-HT turnover in the CNS.

The hippocampus, according to Figure 6.10, showed serotonin changes that were more pronounced. Following exposure to the swim stress of the situational reminder, Group 4 exhibited a profound decrease in serotonin concentration and an elevated 5-HIAA concentrations to support the decreased serotonin concentrations. Dunn (1988) has reported similar results. Low serotonin concentration in rats is characterised by hyper-arousal and an exaggerated emotional response to a relatively mild stimulus (van der Kolk, 1994).

Pretreatment of animals with selective serotonin reuptake inhibitors (SSRIs), as well as tricyclic antidepressants before exposure to inescapable stressor such as forced swim stress/ situational reminder, prevents the behavioural syndrome of learned helplessness (Bonne *et al.*, 2003). Learned helplessness is also regarded as a valid model of PTSD (Uys *et al.*, 2003).

Decreased serotonin concentrations have also been observed in patients diagnosed with combat-related PTSD, providing a possible explanation for the exaggerated startle response

noted in these patients (Spivak *et al.*, 1999). This may reflect a decreased ability to respond to the situational reminder since the response to the TS was less impressive. Therefore the RS was necessary for propagating the fear-related response (Uys *et al.*, 2003).

In contrast to the Acute Study, the Re-Stress Study showed an elevated hippocampal serotonin concentration 3 days post RS (Group 5). Increased hippocampal serotonergic activity has also been observed in chronically stressed animals (Gamaro *et al.*, 2003).

On 7 days post RS (Group 6), hippocampal serotonin concentration was found decreased as compared to Control (Table 6.5). An overall deficit in serotonin may impair long-term memory resulting in the cognitive disturbances of PTSD (van Praag, 2004) and contributing to the comorbidity of mood disorders, insomnia and depression (Albucher & Liberzon, 2002).

Decreased serotonin concentrations on day 3 post RS, could be attributed to the up regulation of the 5-HT_{2A} receptor by elevated corticosterone concentrations (Toth & Benjamin, 1997). The result of this study however showed a statistically significant decrease in corticosterone concentration. However a previous study conducted at our laboratory (Harvey *et al.*, 2003) has found 5-HT_{1A} receptor density increased on day 7 post RS. It can be speculated that increased 5-HT_{1A} receptor density indicated a decreased 5-HT concentration as noted on day 7 post RS.

This finding suggests that repeated stressors as with the TDS model, may possibly lead to increased behavioural sensitivity that is related to the kindling theory. Kalynchuck and colleagues (2001) have shown that kindled rats have increased hippocampal 5-HT_{1A} receptor density that can be correlated to increased fear. In PTSD it seems that the 5-HT_{1A} receptor system may be both under and over-responsive and both conditions contribute to the symptomatology of PTSD i.e. the hyperarousal, fearfulness and reduced habituation to adverse events (van Praag, 2004).

7.3.4 Noradrenaline concentrations

The PFC plays a prominent role in inhibiting memories that are irrelevant to the stimuli. NA exerts potent actions in the PFC in influencing memory (Berridge & Waterhouse, 2003). Exposure to the situational reminder did not cause a statistically significant change in the NA levels of the Re-Stress Study.

A delayed increase in noradrenaline concentrations was noted in the PFC on day 3 post RS, although statistically insignificant (Figure 6.5). This increase may stimulate the α_1 -receptors within the PFC causing a debilitating effect on memory (Berridge & Waterhouse, 2003) although this is not necessarily detrimental to the animal, since increased NA levels are

associated with higher levels of arousal that serve as important survival mechanisms (Berridge & Waterhouse, 2003). The increased tendency was not maintained and by 7 days post RS NA displayed a tendency towards decreased concentration (**Figure 6.5**) highlighting NA dysregulation in the PFC.

Although chronic/repeated exposure to stressors do not necessarily result in increased NA release, it does result in an increased capacity of the system to release NA by increasing the rate of NA synthesis. CRH appears to participate in stressor-induced up regulation of TH synthesis in the LC under some circumstances (Melia & Duman, 1991). It has been suggested that NA in the LC-NA system may contribute to an anxiolytic action under conditions of stress (Weiss *et al.*, 1994). Behavioural sensitisation includes alteration in NA function (Charney *et al.*, 2000).

In this study there was a statistically insignificant tendency towards a decreased NA concentration in the hippocampus (**Figure 6.8**). Repeated exposure to inescapable stress is associated with an eventual depletion of noradrenaline in the hypothalamus and hippocampus (Weiss *et al.*, 1981). Considerable evidence indicates that noradrenergic function is abnormal in PTSD with elevated urinary excretion of catecholamines being reported in PTSD patients (Bonne *et al.*, 2003).

7.3.5 Dopamine, HVA & DOPAC and the DOPAC/DA ratio in the PFC and hippocampus

Dopamine showed no change of statistical significance in both the PFC or the hippocampus although a decreasing trend is evident.

Empirical studies of dopamine involvement in patients diagnosed with PTSD have been inconclusive (Gelernter *et al.*, 1999). Yehuda and co-workers (1992) reported increased urinary dopamine excretion in patients diagnosed with PTSD as compared to healthy volunteers.

Decreased dopamine concentrations as has been the tendency of this study, compromise the ability to develop effective coping strategies to dealing with trauma (Deutch & Young, 1995). Such a deficit may also contribute to hypervigilance and paranoia and greater susceptibility to trauma-related contextual stimuli (Gelernter *et al.*, 1999).

Rats exposed to uncontrollable stress, such as the stressors of the TDS model, were found to have decreased dopamine levels as compared to unstressed rats for as long as two weeks after the removal of the stressor (Lucas *et al.*, 2004). Those rats had a hypo-reactivity to both aversive and pleasurable stimuli (Lucas *et al.*, 2004).

In this study no alteration of significance was observed in the metabolites of dopamine and in the DOPAC/DA ratio in both brain areas. HVA concentrations were noted decreased immediately after RS. Findings in the literature indicate that chronic stress reduces DA output (Imperato *et al.*, 1992) and increases dopamine turnover in the brain (Murphy *et al.*, 2003).

7.3.6 Summary: Re-Stress Study

Koob (1999) postulated that the CRF-NA-CRF system might essentially be important in making adjustments to behaviour in response to demand. This feed-forward mechanism is particularly vulnerable to dysfunction, thus resulting in possible stress sensitisation. Subsequently, abnormal response to stressors i.e. an absence of anxiety following trauma, as was the finding of the current study, is possible.

This behavioural abnormality is supported by hypocortisolemia and a decreased hippocampal serotonin concentration. Prior to exposure to the situational reminder, corticosterone levels had stabilised at a level not significantly different from basal. Of note worthy significance is that exposure to the situational reminder, which is only a mild stressor in comparison to the TS, caused a profound decrease in corticosterone concentration, indicative of a possible HPA-axis dysregulation. The elevated hippocampal MA levels of the Acute Study were not observed after exposure to the RS.

Increased anxiety became evident on day 3 post RS and more profound by day 7 post RS although corticosterone concentrations returned to normal. On day 7 post RS, rats were extremely anxious, as confirmed by all three determinants. Although only the decreased serotonin concentrations in the hippocampus can be regarded as statistically significant, there was a tendency towards decreased MA levels in both the PFC and the hippocampus when comparison was drawn from day 3 post RS to day 7 post RS.

7.4 Final Synopsis

An inability to appropriately regulate or initiate the stress response has been proposed as a critical factor in the pathophysiology of PTSD (Sullivan, 1999). The TDS model is a putative animal model of PTSD that is useful in the study of the response to a severe stressor followed by a mild situational reminder.

The Triple Stressor (TS) of the TDS model has the capacity to induce stress in rodents. The stress response is evident from the presence of anxiety-like behaviour noted on the EPM and the rapid increase in corticosterone concentrations. Corticosterone released during emotionally

arousing events activated noradrenergic mechanisms on day 3 post TS, resulting in enhanced memory of that event (Ferry *et al.*, 1999).

The increased levels of cortisol bring about an increase in CNS serotonin turnover (Davis *et al.*, 1995) as noted on day 3 post TS. Increased corticosterone concentrations modulate MA activity in the CNS.

It is evident from this study that multiple brain structures are involved in the organisation of response to aversive or stressful stimuli. Marked differences were observed in the response to the TS and RS. In response to the TS a statistically significant increase in corticosterone concentrations was found and the RS (which is only a mild situational reminder) in contrast evoked a statistically significant decrease in corticosterone concentrations.

This is an extremely important observation in the context of the neuro-development of PTSD-like pathology after re-experiencing or flashbacks. Clearly hypocortisolemia is an important contributor to the inability of the body to shut-off the stress response.

This implies that the normal extinction of trauma related memories is not put in place. It is also relevant that anxiety was absent by 7 days post TS, but very severe 7 days post exposure to the situational reminder.

The current data would suggest that profound MA dysregulation is induced by the TDS model. The amygdala is responsible for modulating memory storage in both the hippocampal and caudate nucleus dependent tasks (Ferry *et al.*, 1999). The delayed increase in NA levels, in response to the TS, as noted in the hippocampus on day 7 post RS, are not sufficient to cause anxiety (Berridge & Waterhouse, 2003) as was confirmed by the EPM results of this study. In contrast the response to the RS was found to evoke a trend towards decreased NA concentrations. Repeated exposure to inescapable stress is associated with an eventual depletion of noradrenaline in the hypothalamus and hippocampus (Weiss *et al.*, 1994).

Serotonin concentrations are markedly affected by the TS and the RS. The TS initially evoked decreased 5-HT concentrations, which had increased to a level of significance by day 7 post TS. Increased serotonin activity in the CNS is required for the successful adaptation to stress and the prevention of subsequent learned helplessness (Spivak *et al.*, 1999). The RS has been shown to cause a statistically significant decrease in 5-HT concentrations by day 7 post exposure. Decreased serotonin has been associated with heightened anxiety, aggression and impaired long-term memory (Spivak *et al.*, 1999).

Dopamine systems are well known to play a role in the emotional response to rewarding as well as aversive stimulation (Le Moal & Simons, 1991). In the Acute Study DA was noted significantly elevated. In animal models, the administration of psychostimulants, that increase DA concentrations, post-training has been reported to enhance memory, while drugs that decrease catecholamine levels such as reserpine, have been reported to produce memory impairment (McGaugh, 1989).

After exposure to the RS the concentrations of DA had visibly decreased. Low dopamine concentrations compromise the ability to develop effective coping strategies to dealing with trauma (Deutch & Young, 1995). Such a deficit may also contribute to hypervigilance and paranoia and greater susceptibility to trauma-related contextual stimuli (Gelernter *et al.*, 1999).

This study has shown that the neuroendocrine and behavioural response to acute and repeated trauma are different and that the TDS model maybe a useful model of PTSD to study the neurobiology and the treatment response in relation to our current understanding of this disorder.

7.5 References

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Conclusion

Chapter 8

8.1 Introduction

The TDS model uses acute stressors (TS) of intense severity followed by a mild situational reminder (RS) on day 7 subsequent to the acute stressors. The rationale is that prior exposure to a trauma (TS) is an important risk factor for the development of PTSD and consequent exposure to a mild situational reminder (RS) causes the maintenance of the fear response over time (Uys *et al.*, 2003). Previous findings have indicated that the Time Dependent Sensitisation (TDS) animal model of PTSD displays the most profound PTSD-like symptoms in Sprague-Dawley rats 7 days after being exposed to a situational reminder (Naciti, 2002).

The aims of this study were to:

Determine the difference in the stress response to the Triple Stressor and the situational reminder (RS) in order to further validate the TDS model at various time points during and after the stressors. This was done to characterize the development and progression of stress-related pathology by:

- ❖ Quantifying monoamine (dopamine, noradrenaline and serotonin) concentrations in the hippocampus and pre-frontal cortex (PFC) (biochemical correlates).
- ❖ Determining plasma corticosterone concentrations (neuroendocrine parameter).
- Observing anxiety-like behaviour on the Elevated Plus Maze (EPM) (behavioral analyses). All the above mentioned were correlated to determine if a trend was prevalent.

8.2 Conclusion

This study provides conclusive evidence that:

- ❖ **The Triple Stressor (TS) does induce stress in rodents while exposure to the situational reminder (RS) evokes a qualitatively different response that may be necessary for the maintenance of the stress response over time.**

- ❖ **Hypocortisolemia would appear to be an important response of the TDS model that underlies many neurochemical and behavioural changes evoked by the TDS model.**
- ❖ **The TDS model is bi-directional. This is evident from the presence of both hyper and hypocortisolemia and both the presence and absence of anxiety.**
- ❖ **The TDS model induces monoamine dysregulation.**
- ❖ **The TDS model appears to be a valid model for inducing PTSD-like symptoms in adult male Sprague-Dawley rats.**
- ❖ **The TDS model has potential to elucidate the intricate interplay between the psychology and neuroscience that underlies the complex human response to trauma.**

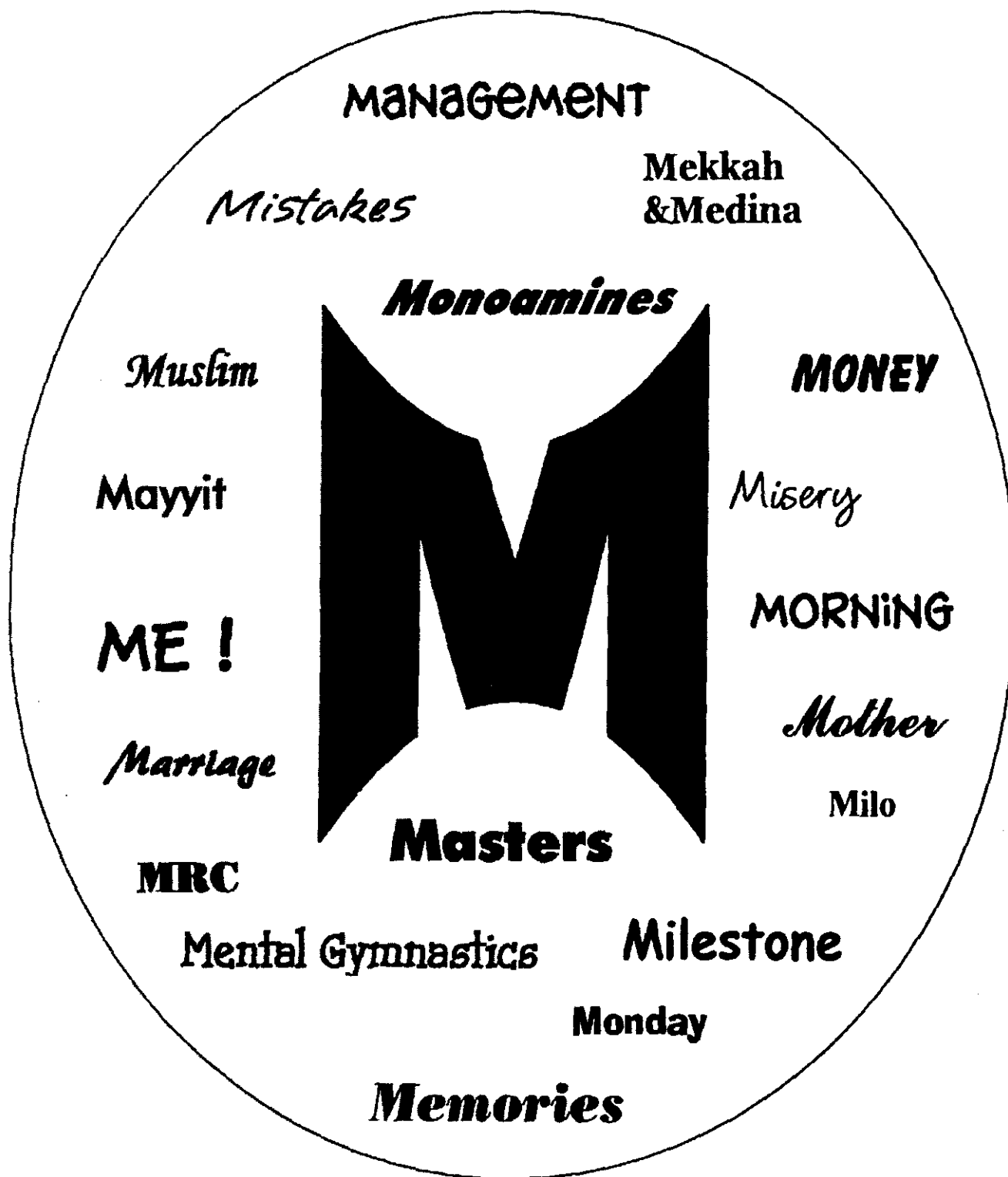
8.3 Prospectus

In recent years, new light has been shed on the complexity and heterogeneity of the clinical picture manifested in PTSD. Pharmacotherapy combined with cognitive therapy is effective in alleviating some symptoms of PTSD thereby encouraging recovery. The future of drug treatment for PTSD holds much promise.

However current agents used in the treatment of this disorder have a distinct shortfall in clinical efficacy, which can be laid at the door of poor understanding of the neurobiological and neuroendocrine correlates that are important in the development of the disorder. Given the interaction between biology and individual experience it is not surprising that the development of pharmacological interventions for PTSD is an urgent challenge.

Further research could clarify the efficacy and utility of pharmacological interventions such as glucocorticoid antagonists, CRF antagonists and ACTH agonists which specifically target the HPA-axis. Severe stress alters complex regulatory pathways through intricate cellular mechanisms thereby contributing to the phenomenology of PTSD. The findings of this study highlight the need for the characterisation of the stress response and continued research in order to integrate and develop effective therapeutic interventions ultimately improving the management of posttraumatic stress disorder.

AFTER THOUGHT.....



“Glory to Thee: of knowledge We have none, save what Thou Hast taught us: in truth it is Thou Who art perfect in knowledge and wisdom”

(The Holy Quraan- Surah 2: Verse 32).

سُبْحَانَكَ اللَّهُمَّ وَبِعَمَلِكَ لَا إِلَهَ إِلَّا أَنْتَ أَسْتَغْفِرُكَ وَأَتُوبُ إِلَيْكَ