INVESTIGATING THE ROLE OF THE NO-CGMP PATHWAY IN AN ANIMAL MODEL OF POSTTRAUMATIC STRESS DISORDER (PTSD)

TANYA BOTHMA

B.Pharm

Dissertation submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

in the

SCHOOL OF PHARMACY, DIVISION PHARMACOLOGY

at the

NORTH-WEST UNIVERSITY (POTCHEFSTROOM CAMPUS)

SUPERVISOR : PROF B.H. HARVEY

POTCHEFSTROOM

OCTOBER 2004

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Abstract

Posttraumatic stress disorder (PTSD) is a severe anxiety disorder characterised by hypothalamic-pituitary-adrenal (HPA)-axis abnormalities, hyperarousal, anxiety, flashbacks of trauma memories and avoidance. Increasing evidence is now accumulating that the disorder is also associated with shrinkage of the hippocampus and cognitive dysfunction that may have its origin in stress-induced excitotoxicity. Animal studies have indeed highlighted a potential role of the excitotoxic glutamate-nitric oxide (NO) pathway in the stress response. Since PTSD appears to be an illness that progresses and worsens over time after an initial severe traumatic event, this study has used an animal model that emphasises repeated trauma to investigate the effect of stress on hippocampal NO synthase (NOS) activity, the release of the nitrogen oxide metabolites of NO (NO_x), and also the evoked release of cGMP. Furthermore, the modulation and dependency of these responses on glutamate, NO and cGMP activity using drugs selective for these targets, will also be investigated.

Rats (n=10/group) were exposed to repeated stress together with saline or drug administration immediately after the stress procedure and continuing for one week post-stress. The animals were then sacrificed for assay of hippocampal NOS activity, NO_x and cGMP accumulation. Animals received either the glutamate-NMDA receptor antagonist, memantine (MEM;5mg/kg ip/d), the neuronal NOS selective inhibitor, 7-nitroindazole monosodium salt (7-NINA;20mg/kg ip/d), the cGMP-specific PDE inhibitor, sildenafil (SIL;10mg/kg ip/d) or the NF$\kappa\beta$ antagonist, pyrollidine dithiocarbamate (PDTC;70mg/kg ip/d). The latter inhibits the nuclear transcription factor, NF$\kappa\beta$, responsible for inducing the expression of iNOS, while it also appears to mediate the glutamatergic actions on NOS expression.

Stress significantly increased hippocampal NOS activity, as well as significantly increased hippocampal cGMP and NO_x levels. These increases were blocked by pre-treatment with either PDTC or 7-NINA, while memantine was without effect. Sildenafil significantly augmented stress-induced NO_x accumulation, as well as cGMP, although the latter failed to reach significance. 7-NINA and memantine significantly blocked the increase in cGMP evoked by time-dependent sensitisation (TDS)-stress,

with PDTC attenuating this response, but not significantly. Additionally, administration of each drug separately for seven days without exposure to stress, did not evoke significant changes in NO_x levels, compared to the control group. However, significant increases in cGMP levels, compared to the control group, were found with all four drugs.

Repeated trauma therefore activates the NO-cGMP pathway, possibly involving actions on both nNOS and iNOS. The NMDA receptor appears less involved after chronic repeated stress, and may have limited therapeutic implications. Sub-cellular NO-modulation, however, may represent an important therapeutic strategy in preventing the effects of severe stress and in treating PTSD.

KEY WORDS: *PTSD, nitric oxide, time-dependent sensitisation, stress, 7-nitroindazole monosodium salt (7-NINA), memantine, PDTC, sildenafil, glutamate, cyclic GMP (cGMP).*

Opsomming

Post-traumatiese stres sindroom (PTSS) is 'n erge angsversteuring wat deur wanfunksie van die hipotalamus-pituitêre-adrenale (HPA)-as, ooropwekking, angs, terugflitse van die traumatiese gebeure en vermyding gekenmerk word. Toenemende getuienis dui daarop dat PTSS met verkleining van die hippokampus geassosieer word, sowel as met kognitiewe versteurings waarvan die oorsprong tot stres-geïnduseerde neurotoksisiteit herlei kan word. Die potensiële neurotoksiese rol wat die glutamaat-stikstofoksiedbaan (Glu-NO-baan) in die stresreaksie speel, is inderdaad ook deur proefdierstudies bevestig. Aangesien PTSS 'n siektetoestand is wat na 'n aanvanklike erg traumatiese gebeurtenis progressief vererger, is 'n dieremodel van herhaaldelike trauma in hierdie studie gebruik. Die effekte van stres op NO-sintetase-aktiwiteit (NOS-aktiwiteit), die vrystelling van NO-metaboliete (NO_x) en die gestimuleerde vrystelling van sikliese GMP (sGMP) in die hippokampus, is met behulp van genoemde model ondersoek. Die regulering en afhanklikheid van hierdie reaksies op glutamaat-, NO- en sGMP-aktiwiteit is ook ondersoek deur van selektiewe teikengeneesmiddels gebruik te maak.

Rotte (n=10/groep) is aan herhaaldelike stres blootgestel en direk na die stresprosedure met normale soutoplossing (saline) of geneesmiddelbehandel vir 'n periode van een week. Hierna het dekapitasie plaasgevind vir analise van NOS aktiwiteit, NO_x - en sGMP akkumulasie in die hippokampus. Al die rotte is behandel met een van die volgende middels: die glutamaat-NMDA reseptor antagonis, memantien (MEM;5mg/kg ip/d), die selektiewe neuronale stikstofoksied sintetase (nNOS) inhibeerder, 7-nitro-indasool natriumsout (7-NINA;20mg/kg ip/d), die sGMP-spesifieke fosfodiesterase (PDE)-inhibeerder, sildenafil (SIL;10mg/kg ip/d) en die NF$\kappa\beta$ antagonis, pirollidien ditiokarbamaat (PDTC;70mg/kg ip/d). Laasgenoemde inhibeer die nukliêre transkripsiefaktor, NF$\kappa\beta$, wat verantwoordelik is vir die induksie van iNOS-uitdrukking, asook die bemiddeling van glutamaat se effekte op NOS-uitdrukking.

Stres het die NOS-aktiwiteit asook die sGMP -en NO_x-vlakke in die hippokampus betekenisvol verhoog. Hierdie verhoogde vlakke is blokkeer deur voorafbehandeling met PDTC of 7-NINA, terwyl memantien-voorafbehandeling geen effek getoon het nie. Sildenafil het die stres-geïnduseerde vlakke van NO_x en sGMP verhoog, alhoewel laasgenoemde nie betekenisvol was nie. 7-NINA en memantien het die verhoogde vlakke van sGMP wat deur tyd-afhanklike sensitisasie (TDS) veroorsaak is, geblokkeer, terwyl PDTC hierdie respons onderdruk het, maar nie betekenisvol nie. Toediening van elke geneesmiddel apart vir sewe dae sonder enige blootstelling aan stres, het nie enige betekenisvolle veranderinge ten opsigte van die kontrolegroep, in NO_x-vlakke teweeggebring nie. Hierteenoor, is betekenisvolle verhogings ten opsigte van die kontrolegroep in sGMP-vlakke vir al vier geneesmiddels aangetoon.

Herhaaldelike traumatiese gebeurtenisse aktiveer dus die NOS-sGMP-baan deurdat dit 'n moontlike invloed op beide nNOS en iNOS uitoefen. Dit wil voorkom of die NMDA-reseptor in 'n mindere mate betrokke is na kroniese herhaaldelike stres wat moontlike terapeutiese implikasies beperk. Sub-sellulêre regulering van NO kan egter 'n belangrike terapeutiese strategie verteenwoordig waartydens die effekte van erge stres voorkom kan word en dus by die behandeling van PTSS gebruik kan word.

KERNWOORDE: *PTSS, stikstofoksied (NO), tyd-afhanklike sensitisasie, stres, 7-nitro-indasool natriumsout (7-NINA), memantien, PDTC, sildenafil, glutamaat, sikliese GMP (sGMP).*

Acknowledgements

I wish to thank our Heavenly Father for all that he has blessed me with and for giving me the knowledge and strength to successfully complete my study.

I wish to express my sincere appreciation to the following people:

Prof. Brian Harvey, my supervisor, for his continuous support, patience and valuable insights as excellent researcher.

Ané Nel, for her encouragement, friendship and practical assistance with the animal model and assays.

The **MRC** and **NRF** for funding, which made this project possible.

All the personnel at the Animal Research Centre, especially **Dr. Douw vd Nest, Antoinete Fick** and **Cor Bester.**

Prof. Faans Steyn, for his statistical consultation services.

Mr Naas van Rooyen and **Mrs Petro Bergh**, for obtaining the necessary chemicals.

Sharlene Nieuwoudt and **Francois Viljoen** as well as all my colleagues at the Division of Pharmacology for their kindness and encouragement throughout this study.

My parents, **Dries** and **Lynette,** I am especially grateful for your unconditional love. Your continuous motivation, understanding and advice have carried me through.

Andrew, for your dedicated help, support and love during the entire study, as well as **Dries**, **Hentie** and **Sanette** for your love and constant encouragement.

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Abbreviations

AADC	L-amino acid decarboxylase
AC	Adenylyl cyclase
Ach	Acetylcholine
ACTH	Adrenocorticotrophic hormone
AD	Alzheimer's disease
Ado	Adenosine
AG	Aminoguanidine
ALAT	Alanine aminotransferase
ALB	Anxiety-like behaviour
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
Amy	Amygdala
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AR	Androgen receptor
ASD	Acute stress disorder

ASR	Acoustic startle response
ATP	Adenosine 5-triphosphate
BBB	Blood brain barrier
BDNF	Brain-derived neurotropic factor
BH_4	Tetrahydrobiopterin
BPD	Borderline personality disorder
BSA	Bovine serum albumin
BST	Bed nucleus of the stria terminalis
BSTL	Lateral bed nucleus of the stria terminalis
Ca^{2+}	Calcium ions
CaM	Calmodulin
CaMKK	Ca^{2+}/calmodulin-dependent kinase kinase
cAMP	Cyclic adenosine 3',5'-monophosphate
CGK	cGMP-dependent protein kinases
cGMP	Cyclic guanosine 3',5'-monophosphate
Cl^-	Chlorine ion
CNG	Cyclic nucleotide-gated
cNOS	Constitutive nitric oxide synthase

CNS	Central nervous system
CO	Carbon monoxide
COMT	Catechol-*O*-methyltransferase
COX	Cyclooxygenase
CPR	Cytochrome P_{450} reductase
CR	Conditioned response
CREB	Brain derived neurotrophic factor
CRF	Corticotrophin-releasing factor
CS	Conditioned stimulus
CSF	Cerebrospinal fluid
Cu^{2+}	Copper ion
CysNO	S-nitroso-L-cysteine
DA	Dopamine
DBH	Dopamine-β-hydroxylase
DCIC	3,4-dichloroisocoumarin
DHEA	Dehydroepiandrosterone
DNA	Deoxynucleic acid
DOPAC	3,4-Dihydroxyphenylacetic acid

DRN	Dorsal raphe nucleus
DXM	Dexamethasone
E	Epinephrine
EAA	Excitatory amino acid
EAAT	Excitatory amino acid transporter
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene glycol-bis[b-amino-ethyl ether]-N,N,N',N'-tetra acetic acid
EMDR	Eye movement desensitization and reprocessing
EMG	Electromyographic
eNOS	Endothelia nitric oxide synthase
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GABA	γ-amino butyric acid
GABA-RC	GABA-receptor complex
GABA-T	γ-amino butyric acid transaminase
Gad	Glutamic acid decarboxylase

GAD	Generalized anxiety disorder
GAF	Guanylyl cyclase-activating factor
Glu	Glutamate
GR	Glucocorticoid receptor
GSNO	S-nitrosoglutathione
GTN	Glyceryl trinitrate
GTP	Guanosine 5'-triphosphate
G-6-PHD	Glucose-6-phosphate dehydrogenase
G-proteins	Guanine nucleotide binding proteins
H_2O	Water
H_2O_2	Hydrogen peroxide
HB	Homogenising buffer
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
HPA	Hypothalamic-pituitary-adrenal
HVA	High-voltage-activated
5-HT	Serotonin / 5-hydroxytryptamine
5-HTP	5-Hydroxytryptophan
ICU	Intensive care unit

IEG	Immediate early gene
iGluR	Ionotropic glutamate receptor
I-$\kappa\beta$	Inhibitory factor kappa B
IL-1	Interleukin-1
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphosphate
ip	Intra peritoneal
K^+	Potassium ion
KA	Kainate receptor
L-ADMA	N^{ω}-N^{ω} dimethyl-L-arginine
LC	Locus coeruleus
LC/NE	Locus coeruleus/norepinephrine
L-DOPA	L-hydroxyphenylalanine
L-NA	N^{ω}-nitro-L-arginine
L-NAA	N^{ω}-amino-L-arginine
L-NAME	N^{ω}-nitro-L-arginine-methyl ester
L-NIO	N-$^{\delta}$ iminoethyl-L-ornithine
L-NMMA	N^{ω}-monomethyl-L-arginine

LPS	Lipopolysaccharide
L-SDMA	N^{ω}-$N^{\omega'}$-dimethyl-L-arginine
LTD	Long-term depression
LTM	Long-term memories
LTP	Long-term potentiation
LVA	Low-voltage-activated
LY-835,83	6-Anilinoquinoline-5,8-quinone
MAO	Monoamine oxidase
MB	Methylene blue
MDD	Major depressive disorder
MDE	Major depressive episode
MDRPG	Multi-drug-resistant 1a-P-glucoprotein
metHb	Methaemoglobin
Mg^{2+}	Magnesium ion
mGluR	Metabotropic glutamate receptor
MHPG	4-Hydroxy-3-methoxyphenylglycol
MK-801	Dizocilpine
MRI	Magnetic resonance imaging studies

mRNA	Messenger ribonucleic acid
MR	Mineralocorticoid receptor
MRS	Magnetic resonance spectroscopy studies
MWM	Morris water maze
Na^+	Sodium ion
Na_2CO_3	Sodium carbonate
$Na_2HPO_{4.2}H_2O$	Di-sodium hydrogen phosphate (hydrate)
$NaH_2PO_{4.2}H_2O$	Sodium di-hydrogen phosphate (hydrate)
NAA	*N*-acetyl-aspartate
N-ac-CysNO	S-nitroso-N-acetyl-L-cysteine
$NaCl^-$	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide
NANC	Non-adrenergic non-cholinergic
NaOH	Sodium hydroxide
N-CAM	Nuclear cell adhesion molecule
NE	Norepinephrine
NEDA	N-(1-naphthyl)ethylenediamine hydrochloride

NF $\kappa\beta$	Nuclear factor kappa B
$(NH_4)_2SO_4$	Ammonium sulphate
7-NI	7-Nitroindazole
7-NINA	7-Nitroindazole monosodium salt
NMDA	*N*-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_2^-	Nitrite
NO_3^-	Nitrate
N_2O	Nitrous oxide
NOS	Nitric oxide synthase
NPR	Natriuretic peptide receptors
NPY	Neuropeptide-Y
NRT	Norepinephrine re-uptake transporter
NS2028	oxidiazolo(3,4-d)benz(b)(1,4)oxazin-1-one
NTF	Neurotrophic factor
O_2	Molecular oxygen

O_2^-	Superoxide
$O_2^{\bullet-}$	Superoxide anion
OCD	Obsessive compulsive disorder
ODQ	1H[1,2,4]oxidiazolo[4,3,-a]quinoxaline-1-one
$OH^\bullet$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
oxyHb	Oxyhaemoglobin
PCPA	para-Chlorophenylalanine
PD	Panic disorder
PDE	Cyclic nucleotide phosphodiesterase
PDTC	Pyrollidine ditiocarbamate
PET	Positron emission tomography
PFC	Prefrontal cortex
pGC	Particulate guanylyl cyclase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
PLA_2	Phospholipase A2

PLC	Phospholipase C
PNMT	Phenylethanol-amine-*N*-methyltransferase
ps	post stress
PTSD	Posttraumatic stress disorder
PVH	Paraventricular hypothalamus
PVN	Paraventricular nucleus
rCBF	Regional cerebral blood flow
REM	Rapid eye movement
ROS	Reactive oxygen species
SEM	Standard error of the mean
SERT	Serotonin re-uptake transporter
sGC	Soluble guanylyl cyclase
SGRI	Selective GABA re-uptake inhibitor
SHR	Steroid hormone receptor
SIN-1	3-morpholinosydnonimine
SNAP	S-nitroso-*N*-acetyl-DL-penicillamine
SNP	Sodium nitroprusside
SSADH	Succinic semi-aldehyde dehydrogenase
SSRI	Selective 5-HT re-uptake inhibitor

TCA	Tricyclic antidepressant
TDS	Time-dependent sensitisation
TH	Tyrosine hydroxylase
TNF	Tumour necrosis factor
US	Unconditioned stimulus
VIP	Vasoactive intestinal polypeptide
VSC	Voltage-sensitive ion channels
VSCC	Voltage-sensitive calcium channels
VTA	Ventral tegmental area

Introduction

Posttraumatic stress disorder (PTSD) is a clinical syndrome that may develop following extreme traumatic stress, and is associated with heightened arousal and a profound increase in autonomic responses, especially related to cardiovascular reactivity (APA, 1994). Consequently, rats subjected to stress present with an increase in atrial sensitivity to circulating catecholamines (Tanno *et al.*, 2002). Of particular interest, in this regard, is that NMDA receptor blockade prevents stress-induced sudden death in cardiomyopathic hamsters (Matsuoka *et al.*, 2002), suggesting that the glutamate pathways mobilised during severe stress may also be driving the peripheral autonomic manifestations of stress. Turning to another key behavioural manifestation of PTSD, viz. deficits in explicit memory, severe stress in animals (Harvey *et al.*, 2003), and patients suffering from PTSD (APA, 1994), display significant cognitive changes.

The nitric oxide (NO)-cGMP signal transduction system has emerged in recent years as a ubiquitous pathway for intracellular and intercellular communication. NO is a simple, but unique, gaseous molecule and free radical, that can serve many diverse functions. Research has demonstrated that the NOS/sGC pathway is coupled to glutamatergic neurotransmission, triggering key events in synaptic plasticity phenomena involved in learning and memory. Furthermore, NO holds great interest because of its apparent role in pathways involved in the response of the brain to severe stress and has been linked to neurodegenerative processes and psychiatric disorders, including PTSD (Ischiropoulos & Beckman, 2003). Hippocampal structural changes and memory dysfunction noted in PTSD have been linked to altered hypothalamic-pituitary-adrenal (HPA-axis) function and the release of glucocorticoids, NO and glutamate (Yehuda *et al.*, 1990; 2000; Sapolsky, 2000). In support of this, pre-clinical studies have now demonstrated the modulatory role that NO exerts on stress-induced behaviour (Masood *et al.*, 2003), and that increased expression of NOS occurs in limbic brain regions following various forms of stress in rats (de Oliveira *et al.*, 2000; Harvey *et al.*, 2004a; Madrigal *et al.*, 2003). This is of particular interest in lieu of the proposed neurotoxic effects of NO in the CNS (Garthwaite & Boulton, 1995) and the evidence of neurodegeneration in animals

subjected to stress. Recent animal studies have also found that cGMP in the hippocampus plays an important role in object recognition memory (Prickaerts *et al.*, 2002). These data suggest an involvement of the NO-cGMP pathway in the hippocampus and that it may have a pathological role in severe stress.

Glutamate and GABA systems are currently attracting a great deal of interest as targets for novel psychotropic drugs (Krystal *et al.*, 2002). Alterations in GABA, and the antidepressant and anxiolytic actions of GABA active drugs, has further stimulated the role of GABA in mood and anxiety disorders and in psychotropic drug action (Shiah-Shin & Yatham, 1998). The importance of glutamate and its association with the NO-cGMP pathway goes much further than simply anxiolytic action. In fact, glutamate and NO-cGMP pathways may hold the key to the presence of neurodegenerative pathology evident in patients suffering form PTSD and depression.

Excessive glutamatergic and nitrergic activity is associated with elevated levels of glucocorticoids and have been implicated in structural remodelling in the brain (McEwen, 1999; 2000) as well as in permanent neuronal damage (Sapolsky, 2000b). Together, the remodelling and eventual damage may underlie the neurodegenerative pathology documented in the hippocampus of patients suffering from severe PTSD (Sapolsky, 2000a). Glutamate pathways are closely associated with cell survival pathways and are key components of synapto- and neurogenesis, neural plasticity and neurodegeneration (D'Sa & Duman, 2002). Of significance is that these stress-induced changes can be reversed by antidepressant treatment, thereby confirming the role of antidepressant-induced neuroplastic events in illness improvement.

Given the important role of NO in the response to glutamate, as well as its neuroprotective/neurotoxic and neuroplastic roles in cell function, the NO-cGMP pathway may represent a valuable new, neurobiological target in the treatment and understanding of PTSD. However, a deeper understanding of the underlying neurobiology of PTSD is needed to enable the development of improved targeted pharmacotherapy.

The current study will investigate the role of NO in stress, as well as the value of pharmacological manipulation of glutamate and NO after exposure of rats to time-dependent sensitisation (TDS)-stress, a putative animal model of PTSD (Uys *et al.*, 2003). Current treatment strategies of PTSD are inadequate and new targets for

improved efficacy are urgently needed. The involvement of NO-cGMP may have great importance in explaining the neuropathological abnormalities characteristic of PTSD, such as anxiety and hippocampal degeneration, but may also open new horizons for pharmacological intervention and treatment of PTSD.

Posttraumatic Stress Disorder (PTSD) Chapter 1

1.1 Introduction

PTSD has been called "shell shock" or "battle fatigue syndrome" (APA, 1994) although its aetiology is complex and multifactorial. Importantly, exposure to a traumatic event does not fully explain the occurrence of the disorder. It has been proposed that stress triggers a cascade of biological events that ultimately lead to the occurrence of chronic PTSD (Segman *et al.,* 2002), while individuals with prior vulnerability are at higher risk for developing PTSD following exposure to a trauma. Previous studies have found correlates of abnormal biological reactivity of the brain endocrine and autonomic nervous system (Segman *et al.,* 2002).

PTSD symptoms usually begin in the first 3 months following exposure to the trauma, however, the appearance of symptoms may be delayed by months or years (APA, 1994). Symptoms that remit within 1 month are recognized as acute stress disorder (ASD). ASD is conceptually similar to PTSD and shares many of the same symptoms. Diagnostic criteria for PTSD include dissociative (emotional numbness, feeling "unreal" or disconnected from emotions or environment), intrusive thoughts, avoidance and arousal symptoms. For a diagnosis of ASD to be met, symptoms must occur within two days and four weeks of a traumatic experience, after which time a diagnosis of PTSD should be considered (Bryant & Harvey, 1997).

If symptoms last only 1 to 3 months, the disorder is diagnosed as acute PTSD (APA, 1994). If these continue after 3 months, it becomes chronic PTSD (APA, 1994). Chronic PTSD is a mental disorder with both psychological and physiological components. Patients may present with somatic complaints and, possibly, general medical conditions, including:

- General appearance may be affected. Patients may appear dishevelled and have poor personal hygiene;
- Behaviour may be altered. Patients may appear agitated and their startle reaction may be extreme;
- Orientation sometimes is affected. The patient may report episodes of not knowing the current place or time;

- Memory may be affected. Patients may complain of forgetfulness, especially concerning the specifics surrounding the traumatic event;
- Concentration is poor;
- Impulse control is poor;
- Speech rate and flow may be altered;
- Mood and affect may be changed. Patients may have feelings of depression, anxiety, guilt, and/or fear;
- Thoughts and perception may be affected. Patients may be more concerned with the content of hallucinations, delusions, suicidal ideation, phobias, and reliving the experience, while certain patients may become homicidal (Gore, 2002).

Individuals with PTSD may be at increased risk for developing panic disorder, agoraphobia, obsessive-compulsive disorder, social phobia, specific phobia, major depressive disorder, and somatization disorder. Over time, untreated and undertreated individuals with PTSD are especially susceptible to experience a deterioration of personal and work relationships and to develop substance abuse/dependence (Gore, 2002).

The likelihood of developing PTSD and the severity and chronicity of symptoms experienced, is a function of many variables, the most important being exposure to a traumatic event. It is therefore important to bear in mind that, even among vulnerable individuals, PTSD would not exist without exposure to a traumatic event (Breslau *et al*, 1998). According to Foa *et al.* (1999), not everyone who is exposed to a traumatic event develops PTSD, however, the following factors appear to trigger and increase the risk:

- Severity and duration of the trauma;
- Proximity to the event;
- The more dangerous it seemed;
- Repetition of trauma;
- Infliction by other people (eg., rape) and negative reactions from family and friends;
- Medical procedures (eg., traumatic birth, intensive care unit stay, awakening during surgery etc.);
- Witnessing the sudden death of a loved one.

People with PTSD also experience emotional numbness and sleep disturbances, depression, anxiety, and irritability or outbursts of anger. Feelings of intense guilt are also common and can lead to further depression and anxiety. Most people with PTSD try to avoid any reminders or thoughts of the ordeal (NIMH, 2003).

Because PTSD is a chronic, devastating disorder for which current treatments are only partially effective (Nutt, 2000), victims are often psychosocially impaired, while the illness appears to get progressively worse over time (Nutt, 2000). Clearly a deeper understanding of PTSD is imperative if we are to improve treatment outcome. Thus, a better understanding of the neurobiology of PTSD and knowledge of the normal mechanisms in the brain responsible for the detection of, and response to, imminent harm, danger or pain, is critical if we are to realise this objective (Nutt, 2000).

This chapter will review the anxiety disorders, but with special emphasis on PTSD.

1.2 Classification of Anxiety disorders

Fear and stress reactions are essential for human survival. They enable people to pursue important goals and to respond appropriately to danger. The stress response (fight, fright or flight) is provoked by a severe threat or challenge and is used to initiate an appropriate action. An anxiety disorder, however, is an excessive / inappropriate aroused state characterised by feelings of apprehension, uncertainty or fear. The anxiety response is often not attributable to a real threat; nevertheless it can still paralyze the individual into inaction or withdrawal. Anxiety disorders also persist, while a healthy response to a threat resolves once the threat is removed. Anxiety disorders are usually caused by a combination of psychological, physical and genetic factors, and have been classified according to the severity and duration of their symptoms and specific behavioural characteristics (Simon *et al.*, 2001):

- Generalized anxiety disorder (GAD), which is long-lasting and low grade;
- Panic disorder (PD), which has more dramatic symptoms;
- Phobias;
- Obsessive-compulsive disorder (OCD);
- Posttraumatic stress disorder (PTSD);
- Separation anxiety disorder (nearly always only in children).

1.2.1 Generalized anxiety disorder (GAD)

GAD is characterised by long-lasting exaggerated and unrealistic worry about such things as health and safety of self and family, finances, work, and chance of accident. This excessive anxiety occurs for at least 6 months, while its physical symptoms cause clinically significant stress or impairment in social, occupational, or other important areas of functioning. Furthermore, anxiety is not due to the direct effects of a substance (e.g. drugs of abuse, medication) or a general medical condition (e.g. hyperthyroidism), and does not occur exclusively during a mood disorder, psychotic disorder, or pervasive developmental disorder (APA, 1994).

1.2.2 Panic disorder (PD) with agoraphobia

Many people experience a panic attack at some time in their lives, but one panic attack does not result in a diagnosis of panic disorder. Panic disorder is characterised by unexpected, repeated episodes of intense fear accompanied by physical symptoms (Table 1-1) (Coffman *et al.*, 2004). At least one of the attacks has been followed by one month (or more) of the following:

- persistent concern about having additional attacks,
- worry about the implications of the attack or its consequences (e.g. losing control, having a heart attack, going crazy),
- significant change in behaviour related to the attacks.

As the frequency of panic attacks increases, the person often begins to avoid places or situations where they fear another attack may occur or where help would not be immediately available (Coffman *et al.*, 2004). This avoidance may eventually develop into agoraphobia, in which the predominant complaint is anxiety. Agoraphobic fears typically involve characteristic clusters of situations that include being outside the home alone, being in a crowd or standing in line, being on a bridge, or travelling on a bus, train or automobile. Most panic attacks occur spontaneously or in response to a particular situation but the frequency of the attacks can vary widely. Recalling or re-experiencing even harmless circumstances surrounding an original attack may trigger subsequent panic attacks (Simon *et al.*, 2001).

1.2.3 Phobia

Phobias can be specific, involving fear of a category of objects (e.g. dogs, heights, snakes) or generalized, where fear occurs in many situations.

- ***Social phobia:*** Also known as social anxiety disorder. This occurs as a marked and persistent fear of one or more social performance situations in which the person is exposed to unfamiliar people. Exposure to the feared social situation almost invariably provokes anxiety which may take the form of a situational bound or situationally predisposed panic attack. This anxious anticipation/distress interferes significantly with the person's normal routine, occupational functioning and social activities. Associated symptoms vary in intensity, ranging from mild and tolerable anxiety to a full-blown panic attack (Table 1-1) (APA, 1994).
- ***Specific phobia:*** This is an irrational fear of specific objects or situations. Specific phobias are among the most common medical disorders. However, most cases are mild and not significant enough to require treatment. The most common phobias are fear of animals, flying, heights, water, injections, public transportation, confined spaces, dentists, storms, etc (Simon *et al.*, 2001).

1.2.4 Obsessive-Compulsive Disorder (OCD)

According to the DSM-IV, OCD is characterised by obsessions or/and inappropriate compulsions (refer to Table 1-1). Distressing or intrusive thoughts and repetitive actions interfere with the individual's daily functioning. These obsessions or compulsions cause marked distress, are time- consuming and interfere with routines and are not part of other co-morbid disorders (APA, 1994).

1.2.5 Depression

Depression is not classified as an anxiety disorder, however, it involves chronic forms of anxiety and stress where the person bears the heavy weight of responsibility for negative events. As with all the disorders – the physical symptoms of anxiety can also be present (Simon *et al.*, 2001).

Further discussion of the anxiety disorders in detail is beyond the scope of this desertation and forthwith only PTSD will be presented and discussed.

Table 1-1: Essential features of different kinds of anxiety disorders (APA, 1994).

GAD	Restlessness or feeling on edge; being easily fatigued; difficulty concen- trating or mind going blank; irritability; muscle tension; sleep disturbance (difficulty falling or staying asleep or restless, unsatisfying sleep).
PANIC DISORDER	During a panic attack a person feels intense fear or discomfort with at least four or more of the following symptoms: Rapid heart beat; sweating; shakiness; shortness of breath; dizziness; nausea; feelings of unreality; numbness; hot flushes or chills; a fear of dying or of going insane.
PHOBIA	***Social phobia*** is manifested by: extreme shyness and discomfort in social settings. Symptoms include: sweating; shortness of breath; pounding heart; dry mouth; tremor. ***Specific phobia:*** When a phobic person confronts the object or situation, he/she experiences feelings of: panic; sweating; difficulty in breathing and has a rapid heart beat.
OCD	***Obsession:*** Recurrent thoughts, impulses or images are experienced as intrusive and cause anxiety / distress. Thoughts, impulses and images are not about real life issues. Person ignores/suppresses/neutralizes thoughts. Person realizes that thoughts are a product of his/her own mind. ***Compulsions:*** Person feels driven to perform repetitive behaviours that aim at preventing/reducing distress.Person realizes that obsessions & compulsions are unreasonable.

1.2.6 Posttraumatic stress disorder (PTSD)

Posttraumatic stress disorder, the subject of this dissertation, is an extremely and usually chronic emotional reaction to a traumatic event that severely impairs social and emotional functioning. PTSD is triggered by violent or traumatic events that are usually outside the norm of human experience, such as experiencing or witnessing sexual assaults, accidents, combat, natural disasters etc. PTSD may also occur in people who have serious illnesses requiring aggressive treatment.

1.3 Epidemiology

As much as 90% of the general population is exposed to a traumatic event during their lifetime (Breslau *et al.*, 1998). Such events include being involved in a life-threatening accident, fire, flood, or natural disaster, serving in combat, being raped,

robbed, or physically attacked, and witnessing the death or injury of another person (Kessler *et al.,* 1995). Approximately 20% of women and 8% of men who have been exposed to such events develop symptoms of PTSD (Kessler *et al.,* 1995). However, the rates are significantly higher for specific traumatic events, for example approximately 65% of men and 46% of women who have been raped develop PTSD (Kessler *et al.,* 1995). The estimated lifetime prevalence of PTSD is 10% in women and 5% in men (Kessler *et al.,* 1995).

The National Comorbidity Survey, which sampled almost 6000 people (aged 15-54 years), found that men were more likely than women to report physical attacks, combat experience, and being threatened with a weapon, held captive, or kidnapped (Kessler *et al.,* 1995). Women were more likely to report rape, sexual molestation and childhood physical abuse (Kessler *et al.,* 1995). Events most commonly associated with the development of PTSD in women were:

- Childhood physical abuse (49%);
- Rape (46%);
- Being threatened with a weapon (33%);
- Sexual molestation (27%);
- Physical attack (31%).

Events most commonly associated with the development of PTSD in men were:

- Rape (65%);
- Combat exposure (39%);
- Childhood physical abuse (22%).

Overall, women were more likely to experience a trauma associated with a high probability of PTSD. In at least 50% of the cases, PTSD symptoms persist over several years (Kessler *et al.,* 1995). The median time to remission among people who seek professional treatment at any time is 3 years; among people who do not seek treatment, it is 5.3 years. More than a third of persons with PTSD have symptoms for more than 10 years (Kessler *et al.,* 1995).

These staggering statistics emphasize the societal burden of the illness, as well as the economic input of the disorder on health care providers and the family.

1.4 PTSD diagnostic criteria

Accumulating evidence suggests that intense psychological trauma can cause long-standing alterations in the neurobiological response to stress (APA, 1994). According to the *DSM-IV™*, the essential feature of PTSD is the development of characteristic symptoms following exposure to an extreme traumatic stressor, through either direct experience, witnessing, or knowledge of an event that involves actual or perceived threat to life or physical integrity. The person's response to the event involves intense fear, helplessness, or horror (APA, 1994). The characteristic symptoms consist of three clusters: persistent re-experiencing of the traumatic event (at least 1 symptom); avoidance of stimuli associated with the trauma, with numbing of emotions (at least 3 symptoms); and increased arousal (at least 2 symptoms) (APA, 1994).

1.4.1 Intrusion / Re-experiencing

Re-experiencing may involve thoughts, memories, perceptions, images or dreams (Bonne *et al.*, 2004). The emotional response is highly stressful and in severe cases people with PTSD may even lose orientation to time and place (i.e. dissociate). Given the association between traumatic recall and seemingly unrelated stimuli and the ensuing fearful response, the mechanism of fear conditioning (refer to section 2.2.1) has often been suggested as a model for the re-experiencing phenomena in PTSD (LeDoux, 2000). In people with PTSD, fear conditioning persists despite absence of threat. Memories of the trauma reoccur unexpectedly and episodes called "flashbacks" intrude into their lives. This happens in sudden, vivid memories that are accompanied by painful emotions that take over the victim's attention. This re-experience, or "flashback," of the trauma is a recollection. It may be so strong that individuals almost feel like they are actually experiencing the trauma again or seeing it unfold before their eyes and in nightmares (APA, 1999). Rapid eye movement (REM) sleep disturbances and nightmares have been suggested to be the hallmark of PTSD (Kilpatrick *et al.*, 1994). The presence of REM sleep disturbances remains equivocal, however, and no profile of sleep disturbances unique to PTSD has yet been established (see Pillar *et al.*, 2000 for review). Nightmares are reported by as many as 75% of PTSD patients (Kilpatrick *et al.*, 1994), which tend to occur earlier in the night, are more frequent and are more often associated with gross body movements than are idiopathic nightmares (Germain & Nielsen, 2003).

1.4.2 Avoidance

Avoidance symptoms affect relationships with others. The person often avoids close emotional ties with family, colleagues, and friends. At first, the person feels numb, has diminished emotions, and can complete only routine, mechanical activities. When re-experiencing the event, the individual may alternate between the flood of emotions caused by re-experiencing and the inability to feel or express emotions at all. Distressful, unavoidable, repeated and intrusive recollections become an "inescapable stressor" leading to a "learned helplessness"-like condition (refer to section 2.2.1). A person with PTSD avoids situations or activities that are reminders of the original traumatic event because such exposure may cause symptoms to worsen. The inability of these people with PTSD to work out grief and anger over injury or loss during the traumatic event, means the trauma can continue to affect their behaviour without their being aware of it. The neurochemistry involved includes neurotransmitters such as serotonin, dopamine, glutamate and GABA, suggesting that depression may become a product of the inability to resolve painful feelings (APA, 1999).

1.4.3 Hyperarousal

Patients with PTSD may act as if they are constantly threatened by the trauma that caused their illness. Due to generalization and cross sensitisation of threatening stimuli, the unsafe environment expands and all secure havens are abolished, leading to unremitting anxiety (Bonne *et al.*, 2004). Hyperarousal and hypervigilance are commonly experienced by trauma survivors suffering from PTSD (Soutwick *et al.*, 1999). They can become suddenly irritable or explosive, even when they are not provoked. They may have trouble concentrating or remembering current information, and, because of their terrifying nightmares, they may develop insomnia. This constant feeling that danger is near causes exaggerated startle reactions (APA, 1999). Chronic physiologic arousal leads to reduced regulation of autonomic reactions to internal and external stimuli and decreased capacity to respond normally to emotional arousal or external stressors. Arousal is influenced by multiple neurotransmitters (e.g. norepinephrine, dopamine, acetylcholine and serotonin) that are simultaneously active in varying degrees and in various brain regions such as the hippocampus, amygdala, nucleus accumbens, hypothalamic nuclei etc. (Southwick *et al.*, 1999). Chronic alterations in arousal systems are likely to be very complex and involve long-term changes in neural function (Southwick *et al.*, 1999). To

compensate for the chronic hyperarousal, the person shuts down behaviourally, avoiding stimuli reminiscent of the trauma, and has numbed emotional responses (van der Kolk, 1997).

1.5 Phases of traumatic stress reactions following a disaster

During a disaster or traumatic event severe stressors induce intense emotional responses that occur in different phases as depicted in the table below.

Table 1-2: Responses or reactions induced by qualifying stressors (Appelbaum *et al.*, 1997).

Impact phase	- People react to protect their own lives and those of others – - Some people respond disorganized and may not be able to protect themselves. - Such disorganized or apathetic behaviour may extend into the postdisaster period so that people may be found wandering helplessly in the devastation afterwards.
Immediate postdisaster phase	- Emotional reactions depend on the individual's perceptions and experience of the different stressor elements. - There is recoil from the impact and the initial rescue activities commence. - Initial mental-health effects may appear (e.g., people show confusion, are stunned, or demonstrate high anxiety levels).
Recovery phase	- A prolonged period of adjustment. - It commences as rescue is completed. - Depends on the extent of devastation and destruction that has occurred.

1.6 Differential diagnosis of PTSD

Several psychiatric disorders may resemble PTSD. For example, when a person experiences symptoms of PTSD in response to a stressor that does not qualify as a traumatic event, the diagnosis of adjustment disorder would be warranted (Rauch & Foa, 2003). This diagnosis can also be appropriate when a person has symptoms following a qualifying traumatic event but does not meet full PTSD diagnostic criteria. Embedded in the diagnosis of PTSD is the notion that avoidance and fear associated with the trauma generalize to many areas of life. If the avoidance and fear is limited to a specific aspect of the trauma or to a specific object or situation, a diagnosis of

specific phobia may be more appropriate. For example, if a person who survived drowning simply avoids swimming and is unaffected in other areas of life, specific phobia would be the appropriate diagnosis. If however, the person avoids swimming, cannot be near a lake, or drive near water, cannot sleep, alternates between numbing and high arousal, and is constantly irritable, a diagnosis of PTSD should be considered (Rauch & Foa, 2003).

Acute stress disorder is a syndrome that occurs within two days to four weeks after experiencing a traumatic event and may help to predict who is at highest risk for developing PTSD. The criteria are very accurate at identifying up to 94% of victims at risk for PTSD, and between 50% and 80% who actually develops PTSD (APA, 1994).

Recurrent intrusive thoughts occur in OCD but are not related to an experienced traumatic event as in PTSD. In OCD, the intrusive thoughts are generally experienced as inappropriate (Cohen, 1998).

Both PD and PTSD involves a significant amount of avoidance in response to feared stimuli, therefore it can be difficult to distinguish between these two disorders. Panic attacks typically occur spontaneously, with no apparent trigger (NIMH, 1994). An individual with panic disorder typically avoids situations in order to prevent the occurrence of a panic attack, where they may fear they are dying from a heart attack or suffering from a respiratory problem, neurological disorder or gastrointestinal condition (NIMH, 1994).

A person with PTSD avoids trauma-related situations in order to prevent the distress associated with the traumatic memory. Flashbacks relating to the original trauma would precede rapid escalation of psychological symptoms (cognitive dysfunction, derealization) and somatic symptoms (dizziness, nausea, sweating, muscle weakness, pounding heart) (Anderson, 2003). Other conditions (e.g. adjustment disorder; depression; panic disorder; substance abuse / dependence disorder) cause many of the symptoms experienced in PTSD.

There is significant overlap between the symptoms of a major depressive episode (MDE) and numbing symptoms of PTSD, however the re-experiencing and avoidance symptoms of PTSD can be an effective way to distinguish these diagnoses. If the patient reports nightmares, repeated thoughts, flashbacks of a

traumatic event, significant avoidance related to the traumatic experience rather than avoidance of activities due to fatique and disinterest, PTSD should be considered.

1.7 Pathophysiology

Several key psychobiologic mechanisms that enable humans to cope successfully with stressful situations function abnormally in PTSD patients (Friedman, 2000). It is widely accepted that a neurochemical imbalance underlies the pathophysiology of mood disorders and recent studies have demonstrated that structural alterations in response to stress, occur in these patients (Manji *et al.*, 2000). Preclinical investigations of learning and memory processes and of neurochemical effects of stress indicate that the neural mechanisms of fear conditioning, extinction, and sensitisation may be operative in PTSD (Charney *et al.*, 1993). The pathophysiology of PTSD may involve dysfunction of several brain structures, particularly the amygdala, locus coeruleus and hippocampus. PTSD patients exhibit abnormal increases in sympathetic nervous system (SNS) reactivity (eg. hyperresponsiveness to normal stimuli) as well as adrenergic dysfunction (elevated urinary catecholamine levels) (Friedman, 2000) together with dysregulation of 5-HT, DA, opiate and HPA-axis neurochemical systems (Charney *et al.*, 1993). Many PTSD patients exhibit increased startle response, an abnormality generally not reported in other psychiatric disorders. Appraisal is a psychological process by which humans evaluate whether a specific situation is potentially dangerous. PTSD patients have lost the capacity of coping, adaptation and survival, and are more likely to appraise neutral situations as threatening (Friedman, 2000).

Forthworth, the brain structures and neurochemical systems involved in PTSD will be discussed in detail.

1.7.1 Neuroanatomy of the stress response

Multiple brain structures are involved in the organization of responses to aversive or stressful stimuli (Figure 1-1). Foremost, among these are the hypothalamus, hippocampus, amygdala, cingulated and prefrontal cortices and hindbrain regions such as the brainstem catecholamine cell body groups (A1/C1; A2/C2; A6) and the dorsal raphe nucleus (Van de Kar & Blair, 1999).

The stress system coordinates the adaptive response of the organism to real or perceived stressors. The main components of the stress system are the hypothalamic-pituitary-adrenal (HPA) axis and the locus coeruleus-norepinephrine / autonomic (LC/NE) pathways (Heuser & Lammers, 2003).

A typical neuroendocrine response involves initially, within seconds, the increased secretion of catecholamines (epinephrine and norepinephrine) from the sympathetic nervous system and adrenal medulla, the release of CRF (refer to section 1.7.2.1.1) and vasopressin from parvicellular neurons into the portal circulation and increased secretion of oxytocin from the neural lobe of the pituitary, and 5-10s later, the secretion of pituitary adrenocorticotropic hormone (ACTH) (Sapolsky *et al.,* 2000). An increased action of catecholamines leads to quick oxygen and glucose delivery to the brain, resulting acutely in enhanced cognitive function (McEwen & Sapolsky, 1995). The secretion of the end product of the HPA system, glucocorticoids, is kept within an optimal range by inhibition of its own release by means of negative feedback through corticosteroid-receptors (Heuser & Lammers, 2003). Glucocorticoids play a very important regulatory role in the neuroendocrine control of the HPA-axis and participate in the control of homeostasis and the response of the organism to stressors (Habib *et al.,* 2001).

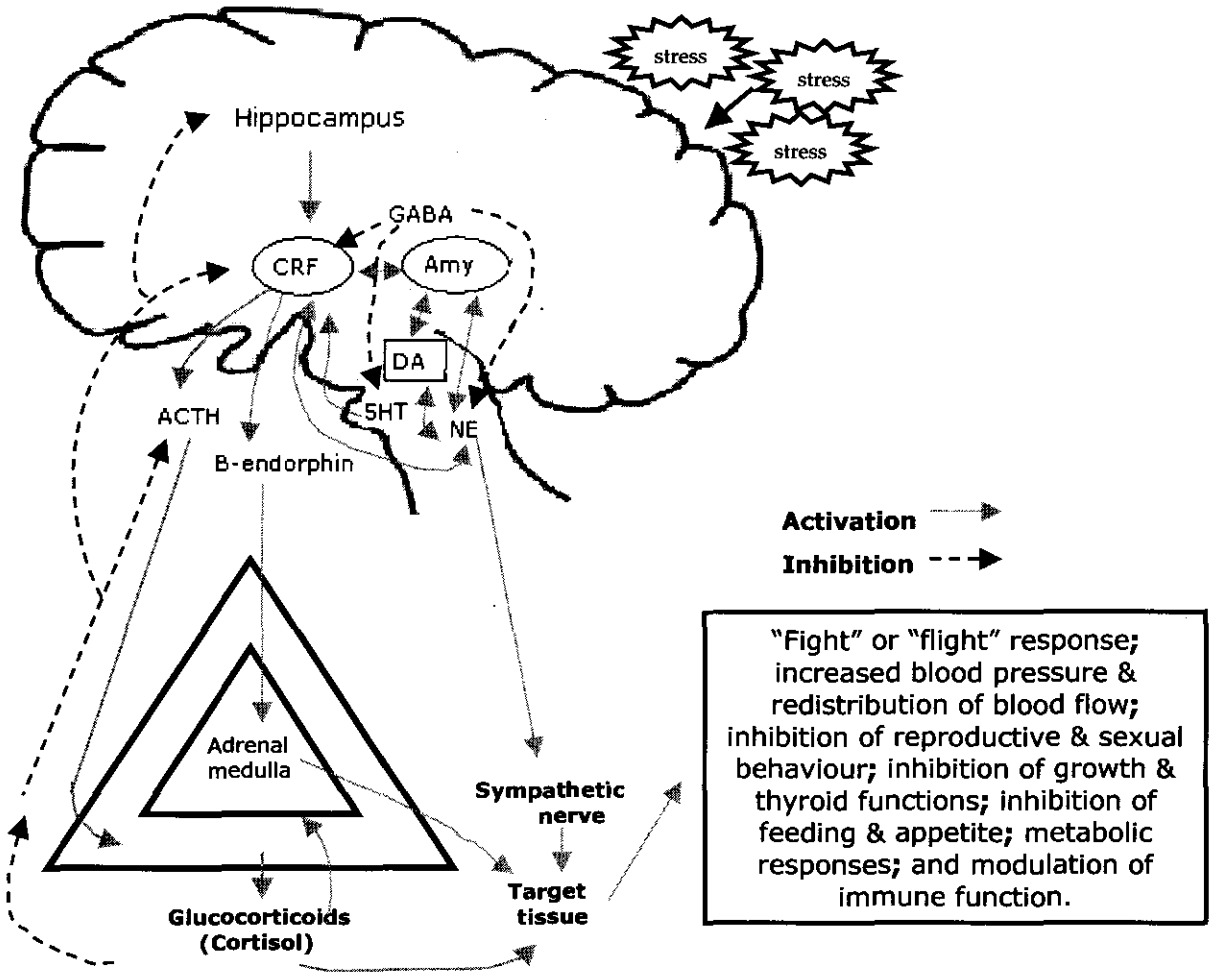


Figure 1-1: Brain circuits participating in the regulation of the neuroendocrine stress response (Carrasco & Van de Kar, 2002). CRF = corticotropin-releasing factor (in the hypothalamic paraventricular nucleus); 5-HT = serotonin (in the dorsal raphe nucleus); NE = norepinephrine (in the locus coeruleus); DA = dopamine (in the mesolimbic system; Amy = amygdala; GABA = γ-gamma-amino-butyric acid.

1.7.2 Neurochemistry

Numerous neurobiological systems are involved in acute and chronic responses to stress. In preclinical and clinical traumatic stress studies, alterations have been reported in the thyroid and HPA-axis, as well as other systems such as the norepinephrine (NE), dopaminergic (DA), epinephrine (E), opiate, GABA and serotonergic systems (Adinoff *et al.*, 1998). Stress causes the levels of opiods (endorphin, enkephalin, etc.) to diminish, creating a sense of urgency that is usually expressed as the need to respond to certain physical demands. The HPA-axis,

consisting of interaction among the hypothalamus, pituitary and cortex, provides a regulatory network linking the brain with the body's behavioural and physiological responses to stress (Adinoff *et al.*, 1998). Hormones, neurotransmitters and neuropeptides are known to interact with one another in a complex fashion so that alteration in one system often affects functioning in other systems (Southwick *et al.*, 1999). For example, in continious stress, the stress signal activates the release of opioids (causing urgency), which then leads to the release of DA (causing high alertness and memory enhancement) and to a decrease in GABA levels (causing anxiety). The latter suppresses serotonin (5-HT) release (causing sleeplessness) and enhances E and NE release which results in hypertension, cardiovascular- and gastrointestinal diseases, etc (refer to Figure 1-1).

1.7.2.1 Adrenocortical hormones

The adrenal medulla is functionally related to the sympathetic nervous system. It secretes the hormones NE and E in response to sympathetic stimulation. The adrenal cortex secretes an entirely different group of hormones, called corticosteroids. These hormones are all synthesized from the steroid cholestrol (Guyton & Hall, 1996). Two major types of adrenocortical hormones, the mineralocorticoids and the glucocorticoids, are secreted by the adrenal cortex. In addition to these, small amounts of sex hormones are secreted, especially androgenic hormones, which exhibit similar effects in the body as the male sex hormone testosterone. The adrenal cortex is composed of three relatively distinct layers. Aldosterone is secreted by the zona glomerulosa, the outermost and very thin layer of cells on the surface. Cortisol and several other glucocorticoids are secreted by the zona fasiculata, the middle layer, and the zona reticularis, the deep layer (Guyton & Hall, 1996).

In response to acute physical or psychological stress, parvocellular neurons of the paraventricular hypothalamus (PVN) produce increased amounts CRF (refer to section 1.7.2.1.1). This is then channelled directly to the pituitary gland (via portal vessels) that is situated just beneath the hypothalamus. Within the pituitary, CRF then stimulates the release of ACTH, that arrives at the adrenal cortex via the bloodstream where it stimulates the secretion of cortisol (Figure 1-2). Cortisol then travels through the bloodstream, exerting effects on multiple organs and tissues (Adinoff *et al.*, 1998). This rapid activation of the HPA-axis causes an increase in

gluconeogenesis, lipolysis, proteolysis and insulin resistance and can be life sustaining because of the metabolic effect of elevating blood glucose levels (Holsboer, 1999). In humans, cortisol levels decrease during the late evening hours, reaching their lowest point during the early mornings. Cortisol secretion begins to increase several hours prior to awakening, and peak levels occur in the late morning hours. In addition, complex short-term fluctuations of cortisol levels occur within the day (Gudmundsson & Carnes, 1997). The HPA-axis incorporates a system of controls that dampen its own activation, the negative feedback system. This system allows direct feedback of cortisol to both the hypothalamus and the anterior pituitary gland to decrease the formation of CRF and ACTH respectively. Both the hypothalamus and the pituitary gland are sensitive to this inhibition by cortisol. Thus, when activation of the stress response produces increases in CRF and ACTH, the resultant elevation in cortisol (after a time delay) suppresses further CRF and ACTH production. Both of these feedbacks decrease the concentration of cortisol in the plasma at times when the body is not experiencing stress. These interactions help to ensure that the body's stress response system does not overreact in its response to a stressor (Guyton & Hall, 1996).

In neurons, circulating corticosteroids may bind to different intracellular receptors, the androgen receptors (AR), mineralocorticoid receptors (MRs) and the glucocorticoid receptors (GRs) (Kerr *et al.*, 1996). The MRs and GRs are co-located in those brain structures involved in the regulation of fear and anxiety, such as the hippocampus (which mediates a variety of effects on neuronal excitability, neurochemistry, and structural plasticity), the septum and the amygdala (Amy) (DeKloet *et al.,* 1998). These receptors differ in their distribution in the mammalian brain, with MRs predominantly localized in limbic areas particularly the hippocampus. Within the hippocampus, the highest density occurs in the CA1 pyramidal cell region (Kerr *et al.*, 1996). GRs are more widely distributed across all brain regions and the highest concentration are found in regions involved in feedback regulation of the hormonal stress response, for example the paraventricular hypothalamus, the hippocampus and the pituitary (Van Haarst *et al.*, 1997). MRs and GRs not only differ in their neuroanatomical distribution, but also in their affinity and binding capacity for corticosteroids. MRs are unique among the steroid hormone receptors (SHRs) in being activated by both cortisol or corticosterone and aldosterone with equal affinity and are almost saturated under basal conditions (Korte, 2001). In fact, MRs have a tenfold higher affinity for cortisol or corticosterone than the GRs, and aldosterone activates the receptor at lower concentrations than are required by cortisol (Rogerson

& Fuller, 2000). Furthermore, due to the greater potency of aldosterone, cortisol (once bound), dissociate much more rapidly than aldosterone from the receptor (Lombés *et al.*, 1994). As mentioned above, GRs have a tenfold lower affinity for cortisol or corticosterone and become occupied only during stress and at the circadian peak (to maintain homeostasis) when glucocorticoids are high (Rogerson & Fuller, 2000). Therefore, after prolonged (acute traumatic) stress MRs are downregulated whereas GRs are increased in number. Similar changes can be observed in patients with PTSD (Haddjer *et al.*, 1998).

There are three types of plasticity in the hippocampal formation in which adrenal steroids play a role. First, adrenal steroids participate along with excitatory amino acids in regulating neurogenesis of dentate gyrus granule neurons in which acute stressful experiences can suppress the ongoing neurogenesis (Gould *et al.*, 2000). Second, adrenal steroids participate along with excitatory amino acids in a reversible stress-induced remodeling of dendrites in the CA3 region of hippocampus (McEwen, 1999). Third, adrenal steroids reversibly and biphasically modulate excitability of hippocampal neurons and influence the magnitude of long-term potentiation (LTP), as well as producing long-term depression (LTD) (DeKloet *et al.*, 1998). These effects on neuronal responses may be involved in biphasic effects of adrenal secretion on excitability and cognitive function and memory during the diurnal rhythm and after stress (Diamond *et al.*, 1996).

The following figure shows the overall system for control of cortisol secretion. The central key to this control is the excitation of the hypothalamus by different types of stress. These stressors activate the entire system to cause rapid release of cortisol, and the cortisol in turn initiates a series of metabolic effects directed toward relieving the damage nature of the stressful state.

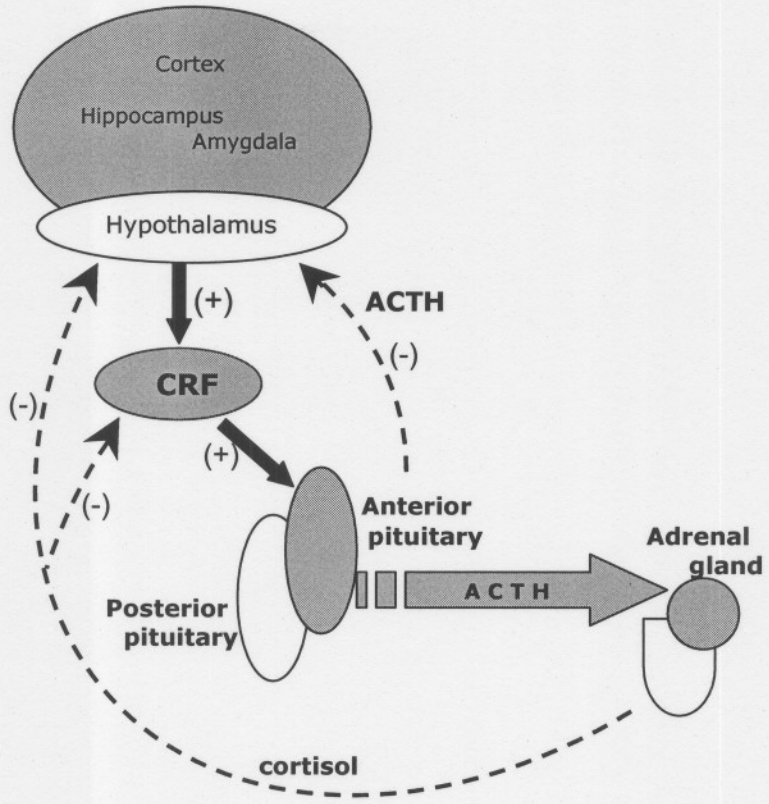


Figure 1-2: The hypothalamic-pituitary-adrenal axis (HPA-axis) (Fitch & Dryden, 2000).

The short activation of the HPA system is known as the "adaptive response", while the "maladaptive response" (refer to section 2.2.1, Figure 2-1) often results from overproduction of stress hormones and/or the failure to terminate HPA activation. Chronic stress can result in sustained increases in glucocorticoids, leading to hyperactivity of the HPA-axis hyperactivity due to an impaired feedback inhibition by the endogenous glucocorticoid, cortisol (Heuser & Lammers, 2003). This feedback is mediated by the GRs and the MRs in the brain (Figure 1-3) (McEwen, 2000). Plasma cortisol cannot freely enter the brain by passive diffusion, because its access is limited by membrane steroid transporters, for example multi-drug-resistant 1a P-glucoprotein (MDRPG) localised at the blood brain barrier (BBB) and possibly in neurones. These transporters capture cortisol from the apical membrane of the endothelial cells of the BBB so that it enters the cells by passive diffusion, while these transporters also expel the hormone back into the plasma (Figure 1-3, dotted arrow) (Pariante *et al.*, 2003). The function of GRs is reduced in depressed patients (GR resistance). Consistent with this, they also exhibit impaired HPA-axis negative

feedback in the context of elevated circulating levels of cortisol (Nemeroff, 1996). Antidepressants reverse these putative GR changes (Pariante *et al.*, 2003). Membrane steroid transporters seem to participate in the regulation of GR-mediated negative feedback and HPA-axis activity, and antidepressants may inhibit these transporters at the BBB and in neurones, so that more cortisol is able to enter the brain (Figure 1-3) (Pariante *et al.*, 2003). This leads to increased activation of brain GRs (and MRs), increased negative feedback on the HPA-axis and, finally, normalisation of HPA-axis hyperactivity in depressed patients.

According to Yehuda (1998), patients with PTSD may involve a HPA-axis that is characterised by enhanced sensitivity to feedback inhibition. Patients with PTSD experience chronic and recurrent stress events that lead to a sustained increase in cortisol secretion at the onset of the trauma, together with a potentiation of GR responsiveness to subsequent stressors and neuronal "hypersecretion" of CRF (Bremner *et al.*, 1997). To protect against the toxic effects of elevated cortisol, the HPA-axis in PTSD becomes increasingly sensitised to feedback inhibition from cortisol through upregulation of glucocorticoid receptors and other mechanisms (Macher & Crocq, 2000). However, the combination of high CRF concentrations and normal or low peripheral cortisol concentrations, appears to be unique among in patients with PTSD (Kasckow *et al.*, 2001). However, it is important to notice that this observation is not definitive, and some controversy exists (Baker *et al.*, 1999; Liberzon *et al.*, 1999).

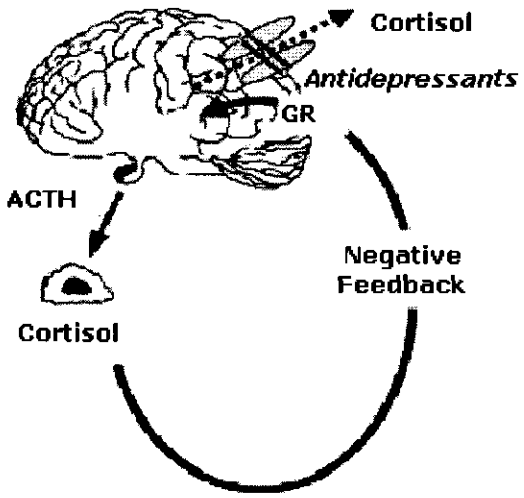


Figure 1-3: A model of the effects of antidepressants on cortisol access to the brain and regulation of HPA-axis function (Pariante *et al.*, 2003).

1.7.2.1.1 Corticotropin-releasing factor (CRF)

The hypothalamic releasing hormone, CRF, controls ACTH secretion through its secretion from the paraventricular nucleus (PVN) of the hypothalamus. This nucleus, in turn, receives many nervous connections from the limbic system and lower brain stem. CRF peptides play a prominent role in mediating the effects of stressors on the HPA-axis and in coordinating the endocrine, autonomic, behavioural and immune responses to stress (Stout *et al.,* 2002; Van de Kar & Blair, 1999). Therefore there has been interest in investigating the potential pharmacological modulation of this system to reduce the maladaptive responding to stressful stimuli, which is characteristic of the anxiety disorders. For example, several studies suggest that antagonizing the CRF_1 and CRF_2 type of receptors result in profound reductions in stress-related behaviours (Ho *et al.,* 2001).

1.7.2.1.2 Cortisol

The body has powerful mechanisms to cope with stress. One mechanism of particular importance is cortisol, without which a human or animal cannot respond appropriately to physical or mental stress. Cortisol, however, affects a wide range of critical physiological processes and therefore must be tightly controlled. Such controls occur via regulation of the HPA-axis (Guyton & Hall, 1996).

Cortisol is classified as a glucocorticoid because of its effect on intermediary metabolism, including the regulation of blood pressure and cardiovascular function as well as regulation of the body's use of proteins, carbohydrates, and fats. Cortisol secretion increases in response to any stress, whether physical (such as illness, trauma, surgery, or temperature extremes) or psychological. When cortisol is secreted, it causes a breakdown of muscle protein, leading to the release of amino acids into the bloodstream and used by the liver for gluconeogenesis. This process raises the blood sugar level. However, when cortisol is produced in excess, the breakdown of protein may lead to muscle weakness and decreased bone mass (Adinoff *et al.,* 1998). At the same time the other tissues of the body decrease their use of glucose as fuel. Cortisol also leads to the release of fatty acids from fat cells, for use by the muscles. Taken together, these energy-directing processes prepare the individual to deal with stressors and insure that the brain receives adequate energy sources. Cortisol has a number of effects that are beneficial to short-term survival, including suppresion of reproductive and immune function, promotion of analgesia, activation of the peripheral autonomic system, suppression of gastric

motility and gastric acid secretion, increases in total oxygen consumption, as well as increases in glucose and glucagon concentrations in plasma (Owens & Nemeroff, 1991).

1.7.2.1.2.1 Cortisol and its role in PTSD

Some studies, but not all (Lemieux & Coe, 1995; Baker *et al.*, 1999) have indicated that PTSD is associated with decreased glucocorticoid levels (below basal levels) and not with an increase in glucocorticoids as would be expected following a stressful event (Yehuda, 1997; Heim *et al.*, 2000). Several mechanisms have also been postulated for the abnormally low cortisol levels in PTSD, including (i) increased sensitivity of the HPA-axis to feedback inhibition by cortisol or (ii) decreased adrenocortical responsiveness (Yehuda, 1997). Patients with PTSD appear to have increased sensitivity of hippocampal glucocorticoid receptor signalling to circulating levels of cortisol, consistent with the hypothesis that increased sensitivity may be critical in mediating hippocampal toxicity in PTSD (Yehuda *et al.*, 1995b; Sapolsky, 2000a). However, recent findings of relatively low density glucocorticoid receptors in the primate hippocampus suggests that the stress-neurodegenerative effects of glucocorticoids may in fact be mediated by high density glucocorticoid receptors in neocortical and hypothalamic areas (Sanchez *et al.*, 2000).

Furthermore, studies in traumatized humans with PTSD have indicated both suppressed plasma cortisol levels and other HPA abnormalities suggestive of a heightened sensitivity of this axis to stressful stimuli (Yehuda *et al.*, 2000). According to Yehuda (1997), a larger number of glucocorticoid receptors have been found in traumatized individuals compared to normal people. Having this, more cortisol is allowed to bind to the receptor (characterised by suppressed glucocorticoid release) and increases the sensitivity of the HPA system. Indeed, this has been confirmed by using the low-dose dexamethasone suppression test (Yehuda *et al.*, 1993 & McFarlane *et al.*, 1997). Evidence, using the time-dependent sensitisation (TDS) stress model, (which purportedly mimics important endocrine responses characteristic of PTSD) also demonstrates hypocortisolemia in test animals (Liberzon *et al.*, 1997; Harvey *et al.*, 2003). This is in accordance with suppressed glucocorticoid levels found in PTSD. Given this hypocortisolemia, the basis for hippocampal neurodegeneration and cognitive decline in patients with PTSD remains unclear.

In chronic PTSD, low cortisol levels are noted in the presence of high catecholamine levels (Yehuda *et al.*, 1997). Thus, catecholamine alterations in PTSD resemble HPA-axis alterations in that both systems appear to be sensitised (Yehuda, 1997).

1.7.2.2 Catecholamines (NE, E, DA)

NE and E are secreted mainly by the adrenal glands and are commonly known as the fight / flight hormones. However, NE is the more important catecholamine concerning the CNS response to stress or trauma (Reist *et al.*, 2001).

Activation of the brain NE system during acute stress is thought to facilitate transmission in many brain regions mediating specific behavioural and physiologic processes comprising the stress response (Ziegler *et al.*, 1999). NE neurons innervate the hypothalamic PVN and have their origin in the caudal nucleus of the solitary tract (A2 cell group) in the dorsolateral medulla, with some contributions from the medullary A1 cell group and the locus coeruleus (LC) (Habib *et al.*, 2001). However, a majority of brain NE neurons are concentrated in the LC.

Through a variety of actions, the locus coeruleus-norepinephrine (LC-NE) system exerts a widespread influence on neuronal circuits and acts as an arousal and alerting system (Aston-Jones *et al.*, 1999b). NE facilitates responding to relevant stimuli while suppressing responding to irrelevant stimuli. In doing so, NE permits the organism to collect and process information most critical to its survival (Berridge & Waterhouse, 2003). The LC-NE system also interacts with the HPA-axis (Cullinan *et al.*, 1995). Evidence suggests CRF inputs to the LC may mediate activation of the LC by physiological stressors, whereas CRF projections from the central nucleus of the amygdala to the LC may activate the LC in response to environmental stressors (van Bockstaele *et al.*, 2001). As such, activation of the LC-NE system may play an important integrative function in coping and adaptation to stress (Koob, 1999). An inability to appropriately initiate or regulate the stress response has been proposed as a critical factor in the pathophysiology of various stress-related disorders (Gold & Chrousos, 1999). The LC-NE system shows pathological changes in a number of neurodegenerative diseases (Marien *et al.*, 2004). Dysregulation of the LC-NE system (specifically NE neurotransmission), has been implicated in stress-related psychiatric diseases such as depression, PTSD and other anxiety disorders and may result in deficits in a variety of cognitive and affective processess (Schatzberg &

Schildkraut, 1995; Sullivan *et al.*, 1999). NE sensitisation presumably may underlie some of the hyperarousal symptoms seen in patients with PTSD (Leonard *et al.*, 2004).

The activity of the LC-NE system is regulated by presynaptic inhibitory α_2-adrenergic autoreceptors (Figure 1-4) (Starke, 2001). By blocking these receptors, α_2 antagonists disinhibit the LC system, leading to an increase in LC neuronal activity and a consequent increase in NE synthesis and release. Concomitantly, α_2 antagonists disinhibit the activity of neuronal and glial targets from the LC-NE system that are negatively influenced by α_2 heteroreceptors. These two actions (increased norepinephrine release and postsynaptic α_2-receptor blockade) synergize to facilitate positive signalling through multiple mediators (Marien *et al.*, 2004).

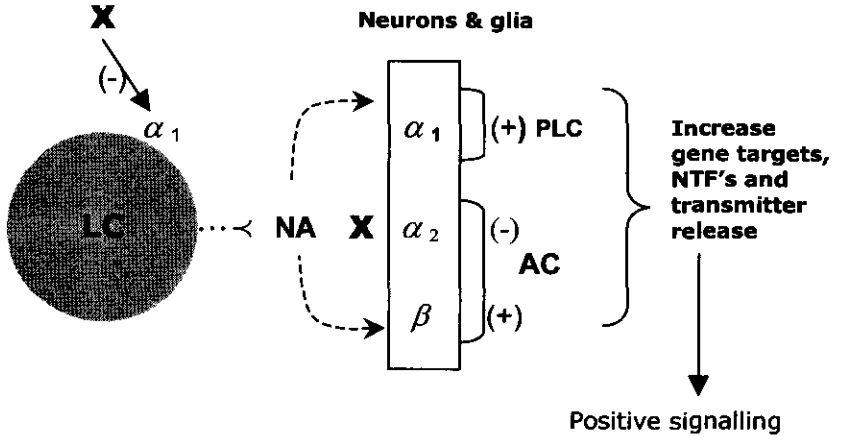


Figure 1-4: Schematic representation of the activation of the LC-NE system by α_2-receptor antagonist "X" (Marien *et al.*, 2004). AC=adenylyl cyclase; PLC=phospholipase C.

Although the LC-NE system innervates virtually the entire CNS, an exception to this is the basal ganglia (striatum and globus pallidus) (Berridge & Waterhouse, 2003). The bed nucleus of the stria terminalis (BST), a component of the extended amygdala, has been implicated in the regulation of neuroendocrine and behavioural responses to stress and arousal (Davis & Shi, 1999; Herman *et al.*, 1994), and receives an extensive norepinephrine innervation, particularly in the medial and ventro-lateral subdivisions (Phelix *et al*, 1992). A recent study showed that acute immobilization stress induces NE release in the lateral bed nucleus of the stria

terminalis (BSTL), and that the release of NE in BSTL facilitated both behavioural and neuroendocrine responses to the acute stress (Cecchi *et al.*, 2002).

Dopamine (DA) (the precursor of NE) also functions as a neurotransmitter in the CNS (Shih *et al.*, 1999). The nigrostriatal-, mesolimbic/mesocortical, and the tuberohypophyseal pathway form the three principle dopaminergic neurone systems in the brain and the various functions of DA are associated with these three pathways (Rang *et al.*, 1999). The LC-NE system gives rise to divergent efferent pathways that provide the major source of NE to the forebrain (Curtis *et al.*, 1997a). Despite a relatively normal baseline firing rate, small perturbations may result in increased release of NE sensitisation while the DA system may also demonstrate sensitisation. DA and E have a central role in the encoding of memory for events and stimuli that are arousing, stressful or fear provoking (Southwick *et al.*, 1999). It has been shown that different stimuli are cross-sensitisers of the DA forebrain, for example in patients with PTSD where hypervigilance and sometimes paranoia occurs (Charney *et al.*, 1993). These symptoms are most likely to be mediated by the DA system.

1.7.2.2.1 Catecholamines and its role in PTSD

Psychophysiological, neuroendocrine, pharmacologic treatment, receptor binding and brain imaging studies provided evidence for increased NE activity in traumatized patients with PTSD (Friedman & Soutwick, 1995; Soutwick *et al.*, 1997). It has also been suggested that altered reactivity of NE neurons is associated with a variety of hyperarousal and re-experiencing symptoms characteristic of PTSD (Southwick *et al.*, 1997).

The autonomic and sympathetic nervous systems are of the most important systems to become activated during stress and threat. The sympathetic nervous system plays a central role in the fight / flight response. In threatening situations this system accelerates the heart rate and increases blood pressure. According to Orr (1997), when PTSD patients are exposed to visual and auditory reminders, heightened sympathetic nervous system arousal is present as well as higher increases in heart rate and blood pressure. A large number of physiologic and biochemical studies also support the notion that traumatized individuals with PTSD have increased responsivity of the sympathetic nervous system that is easily detected when the individual is stressed. Therefore, for some individuals with PTSD, the sympathetic nervous system appears to over respond to a variety of stimuli even years after

having experienced a trauma (Southwick & Yehuda, 1997). This hypersensitivity of the sympathetic nervous system contributes to some of the core symptoms of PTSD, including hyperarousal / exaggerated startle, poor concentration and even intrusive memories (Southwick & Yehuda, 1997).

1.7.2.3 Serotonin (5-HT)

Altering serotonergic neurotransmission through pharmacological manipulation is a complex process involving presynaptic autoreceptors (5-$HT_{1A/1D}$), the 5-HT reuptake transporter (SERT) site and at least fourteen different postsynaptic receptor subtypes (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3) (Figure1-5). Several of these receptors are believed potentially important in mood, anxiety and stress. At the cellular level, abnormalities may include abnormal regulation of 5-HT release and/or reuptake (a role of the presynaptic receptors) or abnormal responsiveness to 5-HT signalling (a role of the postsynaptic receptors) (Kent *et al.*, 2002).

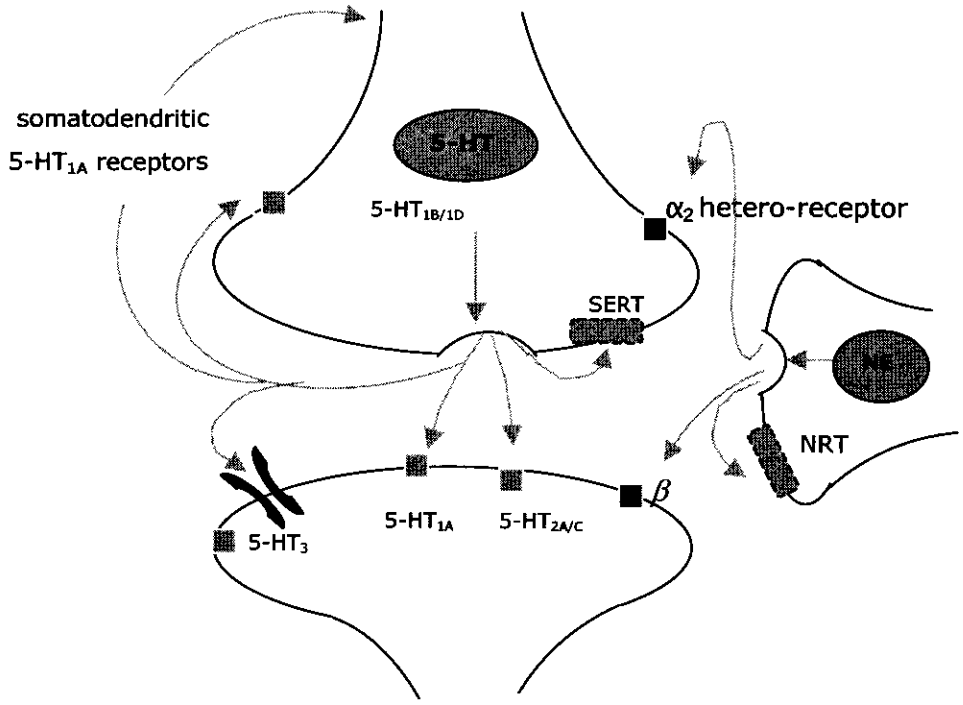


Figure 1-5: Targets within the serotonin (5-HT) synapse (Kent *et al.*, 2002). NRT=norepinephrine reutake transporter; NE=norepinephrine.

Release of 5-HT results in binding to several target receptors, with the resultant action being a summation of effects, including modulation by heteroreceptors, such as the α_2 and β-adrenergic receptors (Figure 1-5).

5-HT is widely distributed in the brain and is involved in mood and impulse control (Fink *et al.*, 1998). Apart from the fact that dysfunction of 5-HT neurotransmission is associated with several mood disorders, serotonergic neurons also have a major influence on the regulation of neuroendocrine function (Best, 1990). 5-HT cell bodies are located in the raphe nuclei located in the brain stem (Figure 1-6) (Baumgarten & Grozdanovic, 1997). In addition, cells containing 5-HT are also located in the area postrema and in the caudal LC, which forms an anatomical basis for a direct connection between the serotonergic and norepinephrinergic systems (Leonard, 1997). Serotonergic neurons located in the midbrain raphe innervate the hypothalamus. Many of these neurons also send collaterals to the amygdala and possibly to other limbic forebrain regions. Thus, changes in the serotonergic input to several limbic forebrain regions can be reflected in changes in the serotonergic input into the hypothalamus (Petrov *et al.*, 1994b).

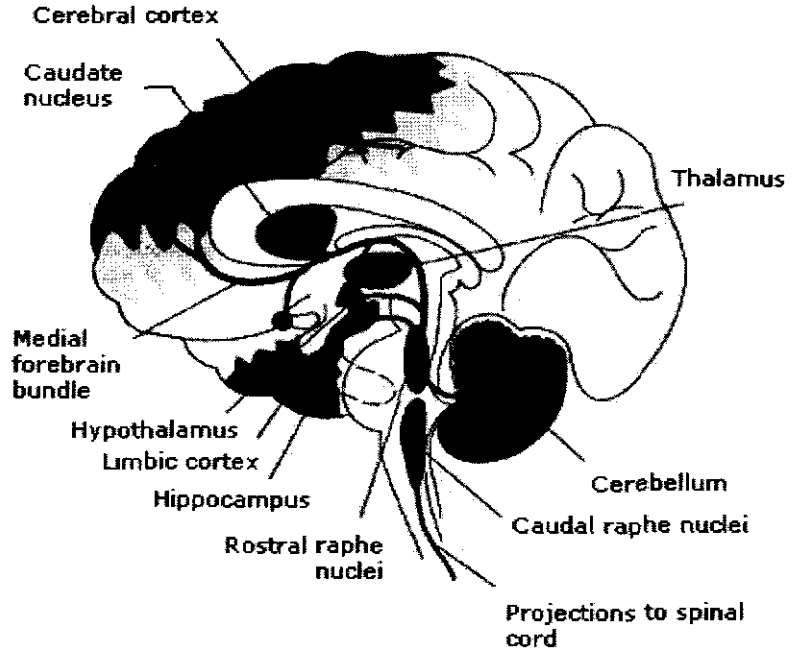


Figure 1-6: Anatomical distribution of the 5-HT pathways in the human brain (Best, 1990).

Administration of 5-$HT_{2A/2C}$ receptor agonists to rats produces an increase in all the hormones that can be classified as stress hormones, including ACTH, corticosterone, oxytocin, prolactin and rennin, while are known to evoke an anxiogenic effect (Barnes & Sharp, 1999). Moreover, 5-HT_{1A} agonists (Barnes & Sharp, 1999) and 5-HT_2 antagonists prevent anxiety (Barnes & Sharp, 1999).

1.7.2.3.1 Serotonin and its role in PTSD

The involvement of serotonin in the expression of PTSD symptoms is suggested by the role of this amine in regulating mood, arousal or sleep as well as by clinical similarity of PTSD and other psychiatric disorders (including depression, anxiety, PD, OCD and eating disorders) which have all been linked to serotonin dysfunction (Graeff *et al.*, 1997). Two examples include the similarity of intrusive thoughts in PTSD and obsessions in OCD and the relation of serotonin to aggression, impulsivity and suicide behaviours that are often reported in combat veterans with PTSD (Markowitz & Coccaro, 1995). 5-HT also plays a consistently important role in brain regions involved in the stress response, especially the PFC and the hippocampus (Harvey *et al.*, 2003).

Experimental studies refer to the association between the serotonergic system and stress conditions, in particular learned helplessness (refer to Chapter 2, section 2.2.2), which is considered as a useful animal model of PTSD (Maier, 1993). This model involves excessive presynaptic 5-HT_{1B} autoreceptor activity as well as decreased 5-HT_{2A} (but not 5-HT_{1A}) receptor density (Wu *et al.*, 1999). Similarly, in the time-dependent sensitisation (TDS) stress model (refer to section 2.3.1) Harvey *et al.* (2003) found a significant elevation in 5-HT_{2A} receptor affinity, which indirectly suggests an increase in 5-HT activity in the prefrontal cortex after TDS-stress. Increased 5-HT activity leads to the release of glutamate and may represent the initial step in the process of neuronal degeneration (Harvey *et al.*, 2004). The TDS-stress model was also found to significantly increase 5-HT_{1A} receptor density (B_{max}) on the seventh day after stress, and significantly reduce ligand affinity (K_d) for this receptor. Thus, TDS-stress produces progressively worsening responsiveness (via sensitisation) of neurobiological mechanisms that mediate synaptic and neuronal plasticity (Harvey *et al.*, 2003).

Supportive findings that alterations in 5-HT metabolism play a role in the pathophysiology of PTSD include the following: Selective 5-HT re-uptake inhibitors

(SSRIs), such as fluoxetine, have beneficial effects in the treatment of PTSD (van der Kolk *et al.*, 1994). Secondly, platelet [^{3}H]-paroxetine binding B_{max} and K_d values are significantly lower in PTSD patients than in normal controls, suggesting a lower number of platelet 5-HT transporters in PTSD patients (Arora *et al.*, 1993). Since the 5-HT transporters play a critical role in 5-HT neurotransmission by reclaiming synaptic 5-HT, Arora *et al.* (1993) have argued that these data reflect pathological changes in 5-HT transporter activity in PTSD. Thirdly, maximal responders to subchronic treatment with fluoxetine had significantly lower pretreatment platelet [3H]-paroxetine binding K_d values than partial responders, suggesting that lower K_d values are possible predictors of SSRI response in PTSD (Fichtner *et al.*, 1994). Of interest is that in the TDS model of PTSD, 5-HT modulating drugs have a profound effect on stress-induced memory and 5-HT receptor changes in limbic regions involved in the stress response (Harvey *et al.*, 2004b).

1.7.2.4 Glutamate pathways

GABA and glutamate are synthesized in the brain from the Krebs citric acid molecule α-keto-glutarate (Figure 1-7). Glutamine is a common precursor for the biosynthesis of both glutamate and GABA.

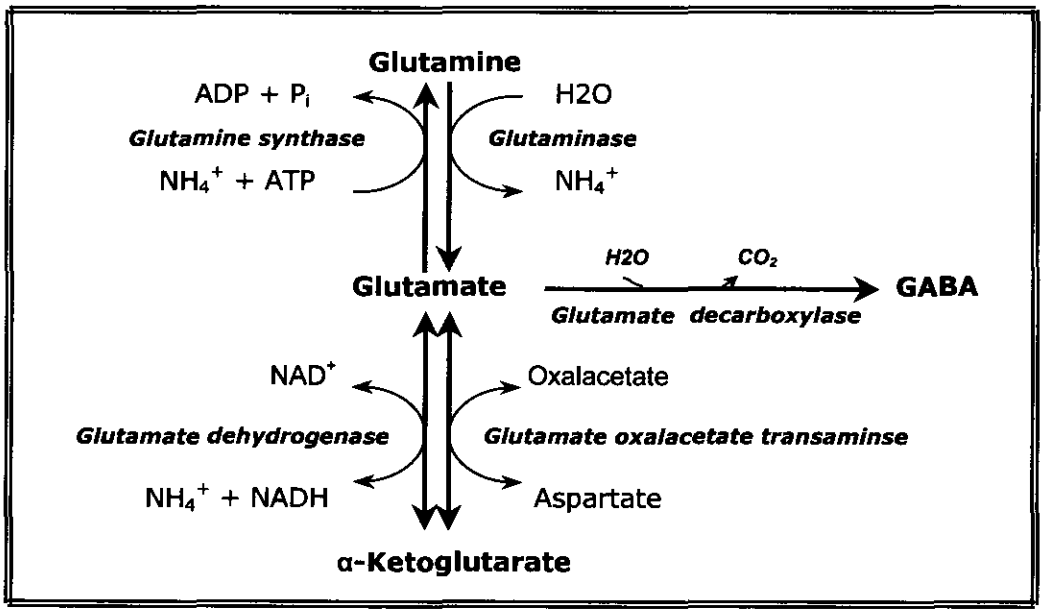


Figure 1-7: Schematic diagram of glutamate synthesis and metabolism and GABA synthesis (Palmada & Centelles, 1998).

Glutamate is the primary excitatory transmitter in the brain and plays an intimate role in the processes of consciousness and memory by mediating sensory inputs to the brain (Collingridge & Bliss, 1995). Among other functions that are dependent on glutamate signalling in the CNS are synaptic development and learning (Skerry & Genever, 2001). Primary sensory transmission, however, involves both glutamate and the inhibitory amino acid GABA.

Expression of different receptor types allows glutamate to activate multiple signal transduction mechanisms, including ionotropic glutamate receptors (iGluRs) and metabotropic receptors (mGluRs). mGluRs are linked to G-protein-dependent second messenger systems, have diverse localizations and functions, and have presynaptic, postsynaptic, heterosynaptic, and glial distributions (Scoepp & Conn, 2002). iGluRs are subdivided into *N*-methyl-D-aspartate (NMDA) receptors which are voltage-dependent cation channels, alpha-amino-3-hydroxi-5-methyl-4-isoxazolepropionic acid (AMPA) receptors which are voltage-independent cation channels and kainate (KA) receptors (Coyle & Enna, 1998). These ionotropic glutamate receptors are associated with opening of sodium- and calcium-permeable ligand-gated ion channels and are generally postsynaptic and mediate fast excitations (Figure 1-8). The AMPA receptors are intimately engaged in the process involved in perception, whereas encoding of factual memory requires coactivation of both AMPA and NMDA receptors (Nutt, 2000).

NMDA receptors are inactive at normal resting membrane potential (-70 mV), because of magnesium ions (Mg^{2+}) that block the channel. mGluRs generally function to modulate postsynaptic excitability to glutamate by providing positive or negative feedback to decrease the release of presynaptic glutamate (Kent *et al.*, 2002). This neuronal depolarization causes the Mg^{+} ions to be removed and therefore activating NMDA receptors. The voltage-dependent cation channels, (associated with NMDA receptors), allow calcium ions (Ca^{2+}) to enter the cell which, in turn, activates a variety of intracellular systems including nitric oxide synthase (NOS) to generate nitric oxide (NO) from L-arginine (Coyle & Enna, 1998). Glutamate also binds to its own transporters termed excitatory amino acid transporters (EAATs), located primarily on glia cells. The EAATs maintain glutamate concentrations in the synaptic cleft and return the transmitter to presynaptic cells for re-cycling and release (Kent *et al.*, 2002).

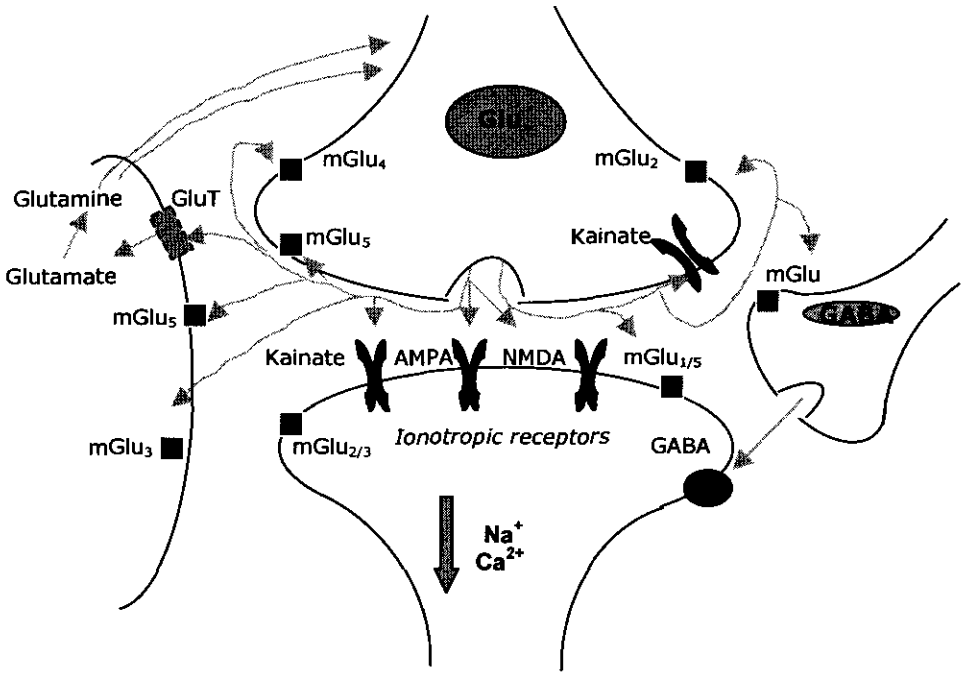


Figure 1-8: Targets within the glutamate (Glu) synapse (Kent *et al.*, 2002). GluT=glutamate reuptake transporter.

1.7.2.4.1 Glutamate and its role in PTSD

In recent years, the role of glutamatergic and NMDA receptor function in the aetiology of PTSD has increased in importance (Dawson & Dawson, 1996; Chambers *et al.*, 1999). Glucocorticoids and repeated stress have been found to induce elevated levels of extracellular glutamate in the hippocampus, as well as increased expression of the glial glutamate transporter, GLT-1 (Harvey *et al.*, 2003). Acknowledgement of the role played by the glutamatergic and GABA pathways in the normal mechanism for encoding memory, leads to the hypothesis that psychological trauma increases glucocorticoid levels which lead to further elevation of glutamate levels and eventuallly cytotoxic cell death. This may be one of the key mechanisms by which brain cells could be lost in chronic PTSD (Vaiva *et al.*, 2004).

Glutamate NMDA pathways in the hippocampus are crucial in memory function (Nutt, 2000), while decreased NMDA receptors are observed in the hippocampus in rats after TDS-stress (Harvey *et al.*, 2003). These changes may underlie cognitive changes previously observed in animals subjected to TDS-stress (Harvey *et al.*, 2003). This suggests that glutamate-modulating drugs may be of value in PTSD.

Indeed, Heresco-Levy *et al.* (2002) has reported the clinical evidence for efficacy of a partial agonist, D-cycloserine, at the glycine regulatory site on the NMDA receptor. Therefore, the NMDA receptor is of great potential importance for the psychobiology and treatment of PTSD (Nutt, 2000).

However, overstimulation of the NMDA receptors following stress leads to long-lasting, excessive influx of Ca^{2+} ions into the postsynaptic neurons (Nutt, 2000). Ca^{2+} binds to calmodulin to activate neuronal nitric oxide synthase (nNOS) that converts L-arginine to NO and L-citrulline. This activation, together with the activation of other Ca^{2+}-dependent enzymes, accounts for many of the deleterious neuronal effects associated with excessive NMDA receptor activation (Almeida *et al.*, 1998). Moreover, studies done on mice by Masood and collegues (2003) indicate that the NMDA-nNOS pathway plays an important role in anxiety-related behaviours, while mice lacking a fully functional glutamate NMDA receptor are less sensitive to stress induced by the elevated plus-maze, light-dark box, and forced swimming tests (Miyamoto *et al.*, 2002). Clearly, the NMDA-receptor cascade may play a pivotal role in the underlying neurobiology of PTSD.

1.7.2.5 γ-Aminobutyric acid (GABA) pathways

Abnormalities in GABAergic neurotransmission have been implicated in numerous psychiatric illnesses, including depression (Sanacora *et al.*, 2000), anxiety, stress and OCD (Shiah-Shin & Yatham, 1998).

In contrast to glutamate, GABA exists as the major inhibitory neurotransmitter in the mammalian CNS. Glutamate stimulation of the NMDA receptor stimulates GABA interneurons to release GABA (Nutt, 2000), with simultaneous release of excitatory and inhibitory neurotransmitters playing a pivotal role in homeostasis. Thus, a fine balance between glutamate and GABA in the brain needs to be maintained in order to prevent excessive levels of excitatory transmission from leading to adverse consequences (Nutt, 2000). GABA receptors have been divided into two main types: ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors (Bloom, 1996). Activation of the $GABA_B$ receptor by GABA causes neuronal membrane hyperpolarization and a resultant inhibition of neurotransmitter release, for example glutamate, to prevent excessive NMDA receptor activation (Kisch, 2002).

The expression of $GABA_A$ receptors is regulated by nitric oxide (Kim & Oh, 2002). Consistent with this, Engelmann *et al.* (2002) have reported that swim stress-induced GABA release in the hippocampus is potentiated by nitric oxide, while $GABA_A$ and $GABA_B$ receptor agonists attenuated stress-induced release on nitric oxide (Ishizuka, 2000). These represent important neuronal mechanisms to curb excess NMDA-NO activity.

1.7.2.5.1 GABA and its role in PTSD

Every person who is exposed to the same stressor may respond differently, with some developing PTSD and others not. This possibly indicates the presence of a mechanism to protect against excessive neurodestructive substances after exposure to a stressful event. Pharmacological probes of GABA activity e.g. β-carbolines, animal models of benzodiazepine-receptor mediated GABAergic function, and the clinical efficacy of benzodiazepines in relieving the re-experiencing and hyperarousal symptoms, all suggest that GABA function may be impaired in PTSD (Bremner & Charney, 1994). In patients suffering from PTSD and in animals subjected to repeated trauma, the protective action of GABA may be impaired (McNally, 1998). In fact, repeated trauma in rats causes an increase in the activation of the nitric oxide synthase (NOS) enzyme, together with an associated attenuation of total hippocampal GABA (Harvey *et al.*, 2004a).

GABA is involved in the process of factual memory registration and in encoding emotional and fear memory (Corcoran & Maren, 2001). GABA is known to modulate the HPA-axis, while it has also been implicated in the pathogenesis of anxiety, depression and insomnia, which are among the disturbances seen in PTSD. Vaiva *et al.* (2004) has indicated that a decrease in post-trauma GABA plasma levels which may represent a predictive factor in the development of acute PTSD.

Thus, a distinct body of evidence supports the role for GABA pathways in the psychobiology of PTSD (Nutt, 2000). The biological disregulation of GABAergic, together with glutamatergic and neuroendocrine pathways, play a fundamental part in the pathology of PTSD and may be responsible for brain structural and functional changes if this devastating disorder remains untreated. GABA pathways also play an important role in regulating normal affective state, and form an integral part of the stress response (Nutt, 2000).

1.7.3 Neuroanatomy of PTSD

1.7.3.1 The Limbic System

The limbic system is a complex set of structures that lies above and around the thalamus, and just under the cerebrum. It includes the hypothalamus, the hippocampus, the amygdala and several other nearby areas (Figure 1-9). It appears to be primarily responsible for our emotional life, and is a vital brain region involved in the formation of memories (Boeree, 2002). Structures near to the limbic system that are immediately connected to it are the cingulate gyrus, septum, ventral tegmental area and the PFC (Figure 1-9). The cingulated gyrus is the part of the cerebrum that lies closest to the limbic system. It provides a pathway from the thalamus to the hippocampus and seems to be responsible for associating of memories to smells and to pain.

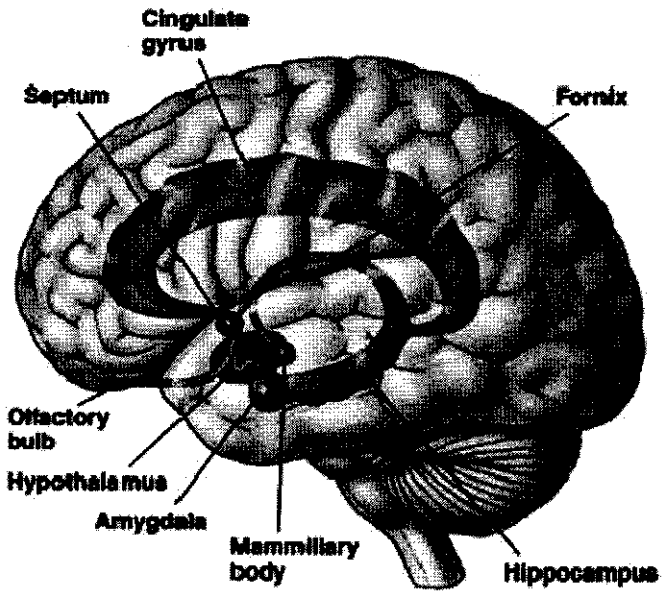


Figure 1-9: Major components of the Limbic system (Mikula, 2003).

The ventral tegmental area of the brain stem consists of dopamine pathways that seem to be responsible for pleasure. The PFC, which is the part of the frontal lobe, is also closely linked to the limbic system, is involved in thinking about the future, making plans and taking action. It also appears to play a part in pleasure and addiction (Boeree, 2002). This group of structures are involved in various emotions such as aggression, fear, pleasure and also in the formation of memory. The limbic system also affects the endocrine system and the autonomic nervous system.

Table 1-3: Important properties of the Limbic system (Amen, 2004).

Functions of the limbic system :	***Problems of the limbic system :***
- Sets the emotional tone of the mind - Filters external events through internal states -Tags events as internally important - Stores highly charged emotional memory - Modulates motivation - Controls appetite and sleep cycle - Promotes bonding - Directly processes the sense of smell - Modulates libido	- Moodiness, irritability, clinical depression - Increased negative thinking - Perceive events in a negative way - Decreased motivation - Flood of negative emotions - Appetite and sleep problems - Decreased / increased sexual responsiveness - Social isolation

The limbic system, along with the temporal lobes, has also been reported to store highly charged emotional memories, both positive and negative (Table 1-3). If a person has been traumatized by a dramatic event, the emotional component of the memory is stored in the deep limbic system. This system also affects motivation and drive. Overactivity in this area is associated with lowered motivation and drive, which is often seen in depression.

In this regard, the hypothalamus is especially responsible for translating emotional state into physical feelings of relaxation or tension. The anterior half of the hypothalamus sends calming signals to the body through the sympathetic nervous system while the posterior half, when stimulated, is responsible for fight or flight response (Amen, 2004).

1.7.3.2 Locus coeruleus (LC)

The cell bodies of most NE neurons in the brain are located within a discrete group of hindbrain nuclei, the most prominent of which is the LC. These NE nuclei are critical in determining the overall state of arousal and attention (Robbins & Everitt, 1995), and serves as a critical component of the brain's alerting or vigilance system (Aston-Jones *et al.,* 1994). Thus acute stress and fear activate the LC-NE system (Jacobs *et al.,* 1995). The meaning, as well as intensity of stimuli, seems to be an important factor in LC responding. Stimuli that signal reward, like those that signal danger, can also activate LC firing (Jacobs *et al.,* 1995). Stress and fear-related activation of the

LC results in increased release of NE in multiple brain regions that are involved in perceiving, evaluating and responding to potentially threatening stimuli. Acute stress-related increases in NE have been found in the amygdala, hippocampus, striatum and PFC. Rapid activation of the LC/NE system facilitates the organism's ability to respond effectively in dangerous situations (Charney *et al.*, 1995). Chronic uncontrollable stress has also been shown to increase responsivity of LC neurons to an exitatory stimulus (Simson & Weiss, 1994). As a result of these and other adaptations, chronically stressed animals may respond to future stressors with exaggerated catecholamine reactivity. It has been hypothesized that enhanced catecholamine synthesis and release may help to protect the organism from depletion of neurotransmitter stores and allow the organism to respond more rapidly and robustly to future stressors, however, in some cases this over-responsiveness may prove to be maladaptive (Southwick *et al.*, 1999).

1.7.3.3 Prefrontal cortex (PFC)

Lesions of the PFC can result in disinhibited behaviour, increased motor activity, impaired attention and diminished ability to inhibit distracting stimuli. Norepinephrine projections from the LC modulate PFC functioning through postsynaptic α_1 and α_2 receptors. It has been suggested that basal release of NE improves PFC cognitive functioning through preferential binding to postsynaptic α_{2A} receptors (Soutwick *et al.*, 1999). After uncontrollable stress when NE release increases above basal levels in the PFC, postsynaptic α_1 receptors become activated causing a decline in PFC functioning (Southwick *et al.*, 1999).

Structural changes and functional deficits have been observed in the medial PFC in patients with PTSD (Zubieta *et al.*, 1999). The PFC (Figure 1-10) plays an important role in planning, guiding and organizing behaviour through working memory, as well as in fear conditioning specifically with regard to extinction of conditioned fear responses. The medial PFC modulates fear responding through inhibitory connections with the amygdala which in turn plays an important role in fear conditioning (Akirav *et al.*, 2001).

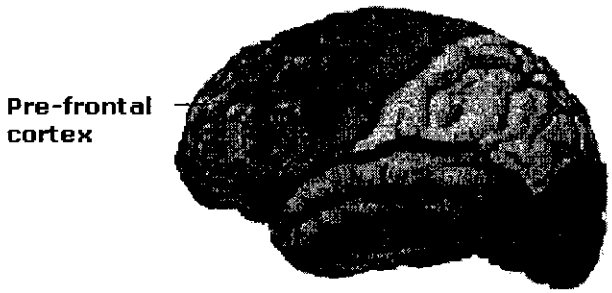


Figure 1-10: The prefrontal cortex (Cardoso, 2003).

1.7.3.4 Amygdala (Amy)

The amygdala (Figure 1-11) consists of two almond-shaped masses of neurons on either side of the thalamus at the lower end of the hippocampus. When it is stimulated electrically, animals respond with aggression. It gives rise to fear and anxiety which leads the animal into a stage of alertness, getting ready for flight or fight (Cardoso, 2003). If the amygdala is removed, animals become tame and no longer respond to things that would have caused rage before, and the animals also become indifferent to stimuli that would have otherwise have caused fear and even sexual responses (Boeree, 2002). The amygdala controls major affective activities like friendship, love and affection and how these alter the expression of mood (Cardoso, 2003). The amygdala and hippocampus, along with the anterior cingulated and medial PFC, are involved in "conditioned fear responses". As information about threat reaches the amygdala, a variety of behavioural and neuroendocrine responses are immediately performed. Information also reaches cortical structures and the hippocampus, which project independently to the amygdala and is essential for modulation and extinction of fear responses (Brewin, 2001).

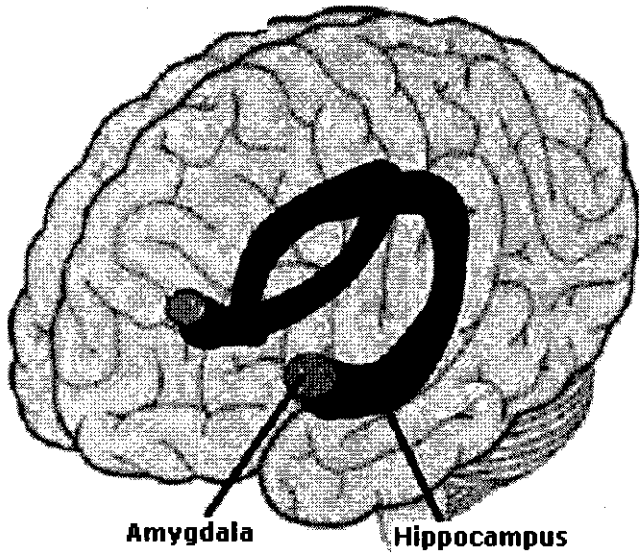


Figure 1-11: The amygdala and hippocampus (Cardoso, 2003).

Highly processed sensory input enters the amygdala so that the elements of a scene that signal danger can be recognized. The association areas of visual, auditory and somatosensory cortices are the main inputs to the amygdala (Weedman, 1997). The amygdala must then be able to control the autonomic system, to provoke an instant sympathetic response to the danger. The main output of the amygdala is therefore to the hypothalamus and brainstem autonomic centres, including the vagal nuclei and the sympathetic neurons (Weedman, 1997).

1.7.3.5 Hippocampus

The hippocampus is located inside the temporal lobe. It forms part of the limbic system and plays a role in memory and navigation (Wales, 2004). The hippocampus consists of two "horns" that curve back from the area of the hypothalamus to the amygdala (Figure 1-11). The hippocampus is critical in converting short-term memory into long-term memory (Cardoso, 2003), such that if damaged, a person cannot build new memories (Boeree, 2002).

The hippocampus is an important brain region regulating stress response in humans and a major feedback site for glucocorticoids It is also highly sensitive to the neurotoxic effects of increased glucocorticoid levels, which are associated with repeated stressful episodes (Sapolsky, 2000a). Loss of hippocampal neurons due to exposure to stress has been reported in animal studies (Sapolsky, 1996) and in

psychiatric disorders that are related to stress events, such as PTSD, borderline personality disorder (BPD), and major depressive disorder (MDD) (Brambilla *et al.*, 2002a; Driessen *et al.*, 2000).

1.7.3.6 How may stress damage limbic brain regions?

Increased levels of glucocorticoids during a stress response lead to hippocampal structural damage in a variety of animal species (Sapolsky *et al.*, 1990). In both PTSD and the TDS-stress model, higher glucocorticoid levels, and the subsequent release of glutamate and NO may heighten the vulnerability of the hippocampus to atrophy (Sapolsky, 2000a). These changes include dendritic remodelling (Woolley *et al.*, 1990), apical dendritic atrophy, alterations in synaptic terminal structure, inhibition of neuronal regeneration (Duman *et al.*, 2001) and neuronal loss (Mizoguchi *et al.*, 1992). Conversely, chronic absence of glucocorticoids can also damage the hippocampus, as demonstrated by the fast apoptotic-like degeneration of rat dentate gyrus cells after removal of the adrenal glands (Sapolsky *et al.*, 1991).

PTSD is a disorder that develops and worsens over time. Therefore it is possible that repeated stress can evoke complex neurobiological responses / changes in the hippocampus, encluding both neurotoxic and neuroprotective pathways. Stress-restress induced downregulation of hippocampal NMDA receptors accompanied by long-lasting increased NOS activity (Harvey *et al.*, 2004a), may result in an increased concentration of intracellular calcium (refer to section 1.7.2.4.1), which ultimately endangers hippocampal cells, produces cyto-skeletal degeneration, protein malfolding and oxygen radical generation (Sapolsky, 2000a). NMDA receptor antagonists block these stress induced dendritic atrophy (Magarinos & McEwen, 1995b).

Serotonin release is also increased in response to stress (Chaouloff, 1993) and may be involved in stress-induced hippocampal damage. Thus, stress-induced atrophy of hippocampal CA3 pyramidal neurons is blocked after administration of tianeptine (Magarinos *et al.*, 1999), a serotonin reuptake enhancer. In this regard, it has been shown that tianeptine could shorten the duration of action of serotonin in the hippocampus (Pineyro & Blier, 1999). A restress-induced 5-HT receptor change is also blocked by the 5-HT-depleter, PCPA (Harvey *et al.*, 2004b).

There is also evidence for interactions between serotonin and NMDA receptors, indicating that serotonin potentiates NMDA receptor binding as well as activity of NMDA receptors, probably via serotonergic (5-HT_2) receptors (McEwen & Magarinos, 2001). Inhibitors of glutamate release also blocks corticosterone- and stress-induced atrophy of CA3 pyramidal neurons, further confirming that glutamate activity via NMDA receptors on hippocampal CA3 neurons may contribute to hippocampal dendritic atrophy (McEwen *et al.,* 1997).

During the last decade, much effort has been directed at determining the molecular and cellular mechanisms of neuronal plasticity, in particular the adaptations underlying learning and memory. These include the cAMP-signalling cascade, involving elements such as the cAMP-dependent protein kinase (PKA), the cAMP-response element binding protein (CREB), and brain derived neurotrophic factor (BDNF) (Egan *et al.,* 2003). This pathway has a pivotal role in neural plasticity and may also be involved in adaptive responses to stress in limbic neural circuits. It has been shown that glucocorticoids modulate the levels as well as the activity of PKA in rat cortex and hippocampus (Dwivedi & Pandey, 2000). Several studies have reported that stress-related behaviours can alter CREB and BDNF in the limbic system and cortex (Bilang-Bleuel *et al.,* 2002). Changes in the PKA, CREB and BDNF were reported after administration of antidepressants (Duman, 2002; Popoli *et al.,* 2002), while abnormalities in the cAMP signalling and related components have also been reported in the post-mortem brain and peripheral cells of patients with affective and anxiety disorders (Chang *et al.,* 2003; Dwivedi *et al.,* 2003).

It is important to note that glial cells, as well as neurons, regulate the metabolism of many molecular factors involved in cellular plasticity, including several neurotransmitters, such as GABA and glutamate (Brambilla *et al.,* 2003). Glia therefore play a critical role in synaptic neurotransmission and the glial cell loss observed in cortical regions of patients with depression, suggests that glial glutamate may also contribute to the necrotic process and cellular death (Bezzi & Volterra, 2001). Two recent studies have suggested that glial cell depletion may contribute to atrophy of brain regions like the PFC and amygdala, through the enhancement of glutamate extracellular efflux (Rajkowska, 2000).

Various studies support the dysregulation of the HPA-axis and glucocorticoid release in PTSD (Yehuda *et al.*, 2000; Yehuda, 1997). Although memory deficits in PTSD may be associated with hippocampal damage (Buckley *et al.,* 2000), small

hippocampal volume and memory deficits could also be pre-existing risk factors increasing the vulnerability to develop PTSD after a traumatic event (Pitman *et al.*, 2001). However, recent work by Vermetten *et al.* (2003) has supported the former hypothesis, with successful treatment of PTSD resulting in improved verbal memory and an increase in hippocampal volume.

Brain imaging findings in PTSD suggest that hippocampal atrophy plays an important role in the pathophysiology of PTSD, with volume reductions in the hippocampus correlated with illness severity and the degree of cognitive deficit (Bremner, 1999). However, these studies do not address the issue of whether smaller hippocampal volumes are pre-existing risk factors for developing PTSD, or induced by trauma (Gilbertson *et al.*, 2002). Magnetic resonance imaging studies (MRI) done by Bonne and colleagues (2001) found no hippocampal volume differences between subjects with and without PTSD at one week and 6 months following traumatic events. In contrast, patients with chronic PTSD where long-standing, unremitted, intense symptoms persist over years or decades, showed hippocampal volumetric reduction due to chronic stress exposure that took a long period to damage hippocampal anatomy (Jerningan & Sowel, 1997). Only a few magnetic resonance spectroscopy studies (MRS) have been conducted in PTSD subjects, showing lower levels of *N*-acetyl-aspartate (NAA) in the hippocampus of patients with PTSD compared to healthy controls. As a result, MRS findings suggest that hippocampal neuronal composition is impaired in PTSD, as NAA reflects changes in neuron viability, density and function. This would be consistent with volume loss of hippocampus reported by MRI studies in PTSD patients (Sala *et al.*, 2004). Positron emission tomography (PET) studies have found regional cerebral blood flow (rCBF) abnormalities in hippocampi of PTSD patients, decreased hippocampal blood flow during retrieval of memories in childhood sexually abused women with PTSD and decreased hippocampal left/right ratio in PTSD individuals with histories of substance abuse (Semple *et al.*, 1993).

1.8 Treatment of PTSD

1.8.1 Psychotherapy

According to the Guidelines of the Expert Consensus Panel for PTSD (Foa *et al.*, 1999), psychotherapy is the first-line treatment choice for acute and chronic PTSD.

Psychotherapeutic approaches include exposure therapy, cognitive therapy and anxiety management. In more severe cases, a combination of antidepressant medication and psychotherapy may be used. Various other psychological approaches have been tried in the treatment of PTSD, for example eye movement desensitisation and reprocessing (EMDR), which is a desensitisation exposure therapy with a cognitive component and hypnotherapy (to treat trauma-related distress) (Foa & Meadows, 1997). Psychodynamic therapy, which focuses on concepts such as denial and stages of recovery from trauma, has also been used in the treatment of PTSD, although methodological flaws have made its efficacy difficult to evaluate (Foa & Meadows, 1997). EMDR, hypnotherapy and psychodynamic therapy, were not rated highly for the treatment of PTSD by the Expert Consensus Panel (Foa *et al.*, 1999).

Exposure therapy is designed to correct the mechanisms underlying PTSD, to help a patient to reorganize the memory, and to neutralize environmental cues (Foa & Meadows, 1997). During such therapy, patients confront their fears (situations, people, objects, memories or emotion) for varying amounts of time in imaginal and/or in vivo situations. Through this procedure, patients come to realize that:

- being in a safe situation that reminds them of their trauma, is not dangerous;
- remembering the trauma is not the same as re-experiencing it;
- anxiety can decrease without avoidance; and
- experiencing PTSD symptoms does not necessarily lead to loss of control.

Cognitive therapy teaches patients to recognize upsetting thoughts, weigh the evidence for and against them, and to adopt more realistic thoughts and behaviours (Foa *et al.*, 1999).

1.8.2 Pharmacotherapy

To date most pharmacologic agents have been chosen for their efficacy in the management of adjunctive symptoms related to PTSD, such as depression and impulsivity, rather than for their effects on PTSD-specific symptoms such as intrusive memories, hypervigilance and increased startle (Southwick *et al.*, 1999).

Pharmacotherapy is useful in reducing or eliminating core and secondary symptoms of PTSD. It also may help patients to uncover pain and thereby facilitate behavioural

and other forms of psychotherapy (Davidson, 1997). Several classes of agents have been explored in the treatment of PTSD, including antidepressants, antipsychotics, anticonvulsants and anxiolytics. Antidepressants such as selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and some of the newer agents have been shown to be effective in treating PTSD (Davidson, 1997). Anxiolytics, however, have been found to have no effect on PTSD symptoms.

1.8.2.1 Adrenocortical hormone modulators

In lieu of the important role of cortisol in the development of PTSD, it is of great interest whether glucocorticoid modulators may be effective in treating PTSD. While this approach has been attempted in depression (Belanoff *et al.*, 2002), it has not yet been explored in PTSD. However, in the putative animal model of PTSD, TDS-stress, the steroid synthesis inhibitor, ketoconazole (KTCZ) has been found to inhibit neurochemical responses evoked by stress in the brain of this animals (Brand *et al.*, 2004; Harvey *et al.*, 2004b).

Several compounds have been investigated in preclinical models of stress and anxiety, but few have proceeded into clinical development (Kent *et al.*, 2002). Intra-dorsal raphe nucleus (DRN) administration of CRF_2 antagonists dose-dependently block inescapable stress-induced behavioural changes in already established fear in rats, while the CRF_1 antagonists administered in the same manner, do not (Hammack *et al.*, 2003). In another study, administrations of CRF_2 antagonists have consistently produced an anxiolytic effect on animals exposed to various types of stress (Takahashi *et al.*, 2001). These promising data , however, needs to be taken into the clinic.

1.8.2.2 Catecholamine modulators

Noradrenergic alterations in PTSD suggest that pharmacologic agents that specifically target NE hyperreactivity might be usful in the treatment of symptomatic trauma survivors. Clonidine, an α_2-adrenergic receptor agonist that has peripheral and central effects, suppresses release of NE through actions at the presynaptic α_2 autoreceptor. It also has actions at the postsynaptic α_2 receptor. Clonidine has been reported as helpful for symptoms of hyperarousal, hypervigilance, sleep

disruption, exaggerated startle response, nightmares, behavioural irritability, and aggression of children with PTSD (Perry, 1994). However, the α_1-adrenergic antagonist, prazosin, may ameliorate nightmares in PTSD (Raskind *et al.,* 2000).

Treatment with betaxolol, a long-acting $ß_1$-adrenergic blocker, has been found to decrease anxiety in several PTSD patients (Swartz, 1998). Propranolol as a nonselective β-adrenergic blocking agent has a suppressive effect on NE activity and is effective in treating the re-experiencing and hyperarousal symptoms of PTSD (Southwick *et al.,* 1999). Open treatment trials of propranolol have also been conducted in children with PTSD (Famularo *et al.*, 1990). These trials have reported a decrease in nightmares, explosiveness, exaggerated startle, insomnia and hyperalertness. It is possible that propranolol, if administered before or immediately after a traumatic event, could prevent or diminish the sensitisation of catecholamine systems and associated PTSD symptoms. It is also possible that early administration of propranolol might prevent the overencoding of traumatic memories that results from stress-related increases in E and NE (Famularo *et al.,* 1990).

1.8.2.3 Serotonin modulators

Modulation of the serotonergic system has been a successful strategy for the pharmacologic treatment of the various anxiety disorders, including panic disorder (PD), generalized anxiety disorder (GAD), obsessive compulsive disorder (OCD), posttraumatic stress disorder (PTSD) and social phobia (SP) (Kent *et al.,* 2002). The efficacy of several selective serotonin (5-HT) reuptake inhibitors (SSRIs) in the treatment of PTSD therefore represents a major therapeutic advance and emphasize the important role of 5-HT with the neurobiology of anxiety and stress.

Although the role of serotonin in PTSD has not been systematically investigated, the SSRIs are rapidly coming into use as first-line agents in the treatment of various anxiety disorders and in particular PTSD (Kent *et al.,* 1998). Furthermore, the SSRIs are effective in treating the secondary symptoms of PTSD, especially numbness and avoidance (Davidson, 2000).

In animals, $5\text{-}HT_{1A}$ medication (e.g. buspirone) has shown benefit in reducing the behavioural effects of inescapable shock, which may serve as an experimental model for PTSD (De Montigny & Blier, 1992). However, the use of buspirone to treat PTSD

has been reported only in open-label case reports and case series with some degree of success (Duffy & Molloy, 1994). The exact role of 5-HT in stress remains controversial, especially since increases in 5-HT levels (with SSRIs) increases fear behaviour (Bughardt *et al.*, 2004) while 5-HT release is increased with acute stress. Moreover, a decrease in 5-HT synthesis or an increase in 5-HT uptake decreases stress-related pathology. This controversial situation is highlighted by recent evidence that stress induced neurodegeneration (Vaidya *et al.*, 1999) as well as neuro-receptor changes (Harvey *et al.*, 2004b) are inhibited by agents that suppress synaptic 5-HT levels. Furthermore, SSRI's are partially effective in treating PTSD, suggesting that deeper seated mechanisms are at play.

1.8.3 Alternative treatment

1.8.3.1 Tricyclic antidepressants (TCAs)

As alluded to above, PTSD is quite resistant to antidepressants. Some efficacy for TCAs has been shown, but treatment is often complicated by side-effects (Ballenger *et al.*, 2000). The future role of the TCAs will depend on results from trials comparing TCAs and more recent antidepressants (Rosenberg, 2003). Nevertheless, there have not been many large-scale double-blind controlled studies of TCA's (Laufer *et al.*, 2003), and recent studies have focused on the SSRIs.

1.8.3.2 Anticonvulsants

As a group, the anticonvulsants are the most studied alternative treatment for PTSD (Ford, 1996; Ballenger *et al.*, 2000). The rationale for using anticonvulsants stems from the kindling model and the role of glutamate in this response, which has been implicated in the aetiology of PTSD (Hertzberg *et al.*, 1999; Friedman, 2000). Indeed, the TDS animal model of PTSD is based on a kindling and sensitisation efect and has shown response to anticonvulsants (Khan & Liberzon, 2003). In this regard, lamotrigine appeares to be superior to placebo in a preliminary treatment trial in PTSD, improving re-experiencing and avoidance / numbing symptoms (Hertzberg *et al.*, 1999). Another anticonvulsant, topiramate, that acts to inhibit amygdala AMPA / kainate receptors, stimulate $GABA_A$ neurotransmission and block voltage-gated Na^+ channels, has demonstrated potent anti-kindling properties in rodent models (Wauquier & Zhou, 1996) and has been shown to alleviate some PTSD symptoms,

such as flashbacks and negative thought intrusions (Berlant & van Kammen, 2002). Other anticonvulsants, such as gabapentin and vigabatrin have also shown some benefit (Brannon *et al.*, 2000).

1.8.3.3 Antipsychotics

Antipsychotic medications are also used in the treatment of PTSD and some benefit has been found in case reports (Dillard *et al.*, 1993). The novel atypical antipsychotic drug, risperidone, is useful in controlling specific symptoms such as flashbacks and nightmares (Leyba & Wampler, 1998), irritable aggression (Monnelly & Ciraulo, 1999), and intrusive thoughts and emotional reactivity (Krashin & Oates, 1999). The possible rationale being the role of excessive DA release in PTSD patients suffering from memory deficits (Southwick *et al.*, 1999), hypervigilance and sometimes paranoia (Charney *et al.*, 1993).

1.8.3.4 Opiate antagonists

Opiate antagonists, including nalmefene and naltrexone, may have beneficial effects in reducing flashbacks (Bills & Kreisler, 1993; Glover, 1993).

1.8.3.5 Inositol

Although myo-inositol, a second messenger, has select efficacy in anxiety disorders responsive to SSRIs (Harvey *et al.*, 2002), it has only limited value in the treatment of PTSD (Kaplan *et al.*, 1996).

1.8.4 GABA treatment

GABA-stimulating drugs such as ethanol and benzodiazepines exert some of their effects through suppressing glutamatergic function (Oosthuizen *et al.*, 2005). According to O'Brien & Nutt (1998) a disruption in the glutamatergic pathway can partly induce a coma. Several GABAergic agents (tiagabine, topiramate, valproate, and carbamazepine) have shown evidence of efficacy in PTSD (Kisch, 2002). One of the cardinal features of PTSD is nocturnal awakening associated with vivid and

very frightening nightmares (Kisch, 2002). Tiagabine potentiates CNS GABAergic function through its unique ability to inhibit GABA reuptake at the GAT-1 GABA transporter (Sabirska *et al.*, 1993). Tiagabine is the only currently available selective GABA-reuptake inhibitor (SGRI) and increases extracellular GABA by up to 200% without perturbing normal physiologic control and without increasing total brain GABA (Sybirska *et al.*, 1993).

Pre-clinical data obtained by Harvey *et al.* (2004a) indicate profound suppression of GABA concentrations compared to basal levels in the hippocampus of animals exposed to TDS-stress. These data suggest that severe recurring stress depletes hippocampal GABA, resulting in unopposed glutamate activity that may drive the development of PTSD.

1.8.5 Dehydroepiandrosterone (DHEA)

The adrenal androgen, DHEA, is an endogenous hormone secreted by the adrenal cortex in response to ACTH (Parker *et al.*, 1996).

Various studies have implicated DHEA in psychiatric symptoms associated with PTSD (Kanter *et al.*, 2001; Sondergaard *et al.*, 2002). DHEA reverses stress induced hippocampal atrophy, probably through modulation of excitatory amino acid-induced neurotoxicity (Watanabe *et al.*, 1992a). This is consistent with the finding of Morfin and Starka (2001) that DHEA has potent antiglucocorticoid properties and is also protective against glutamate and glucocorticoid induced neurotoxic hippocampal damage. Furthermore, Wolkowitz *et al.* (1997) have reported that administration of DHEA has positive effects on mood and memory.

1.8.6 Neuropeptide-Y (NPY) modulators

NPY, a neurohormone- and neurotransmitter- polypeptide, closely involved in the regulation of both central and peripheral noradrenergic system functioning, is densely concentrated in brain regions known to be activated by stress, for example the amygdala, hypothalamus (Renshaw & Hinson, 2001), LC, periaquaductal grey and the PFC (Heilig & Widerlov, 1995).

Preliminary clinical data examining NPY levels suggest that NPY may act as a buffer against acute stress, potentially modulating damaging effects of excessive activation of the HPA-axis. As such, the development of NPY modulators may be useful as future agents in the treatment of PTSD (Kent *et al.*, 2002).

In summary, PTSD is a common disorder. Patients may present with somatic symptoms and frequently have a comorbid medical or psychiatric disorder, therefore PTSD may not be easily recognized. Treatment begins with education and supportive counseling to assure the patient that the response to the trauma is normal and that PTSD has a clear biologic foundation. The first-line treatment choice for PTSD is psychotherapy, using anxiety management programs as well as cognitive and exposure therapies. In severe cases, a combination of antidepressant medication and psychotherapy may be used. SSRIs are considered first-line antidepressant treatment.

The prominent role of NO in neuronal toxicity and the important regulatory role for glutamate and GABA in this process, may explain the stress-restress-related hippocampal degenerative pathology and cognitive deficits seen in patients with PTSD (Harvey *et al.*, 2004a).

Chapter 2 Animal models of PTSD

2.1 Introduction

Psychiatric disorders in humans are among the most complex and incapacitating pathological conditions (Overall, 2000). Animal models of human disorders are desirable for several reasons. Firstly, they offer the possibility of simulating a human condition under controlled circumstances; second, in contrast to human disorders, which can be studied only after they become clinically manifest, animal models are observable as they evolve, permitting the study of symptoms as they develop. Third, they allow the testing of pharmacological and other prospective "treatments" that might be difficult in humans (Yehuda & Antelman, 1993).

Animal models can contribute to understanding the mechanisms underlying anxiety disorders and to developing new medications for their treatment (Shekhar *et al.*, 2001; Martin, 1998). Animal models of stress have the potential to provide information about the course and aetiology of PTSD. According to animal literature, different types of stress paradigms (Table 2-1) lead to different biobehavioural consequences and many different factors contribute to differential responsivity to stress (Yehuda & Antelman, 1993). Animal models of PTSD have used intense stressors, aversive challenges, and situational reminders of a traumatic stress in an attempt to model long-term effects on behavioural, autonomic, and hormonal responses seen in humans with PTSD. Examples include:

- Electric shock (Pynoos *et al.*, 1996);
- Stress-restress or time-dependent sensitisation (TDS) (Liberzon *et al.*, 1997);
- Under water trauma (Richter-Levin, 1998);
- Exposure of animals to a predator (Adamec & Shallow, 1993; Cohen *et al.*, 2003).

In considering relevant animal models of PTSD, it is critical to differentiate between factors that can influence the manifestations or course of PTSD and those that are essential for its induction (Yehuda & Antelman, 1993). Understanding the phenomenology and psychobiology of specific cognitive-affective processes may be

relevant to anxiety disorders (Table 2-1). Some of these processes are relevant to several anxiety disorders and others are particularly pertinent to specific conditions (Uys *et al.*, 2003). In humans, these processes may have unique attributes, but it is still possible to study such processes in other animals. Table 2-1 refers to animal models of various disorders that have been used to broaden the understanding of the neuroanatomy, highligting the regions such as the amygdala and hippocampus. The central nucleus of the amygdala plays a critical role in the fear-potentiated startle response because it projects directly to one of the brain-stem nuclei necessary for startle and lesions of this pathway block the ability of conditioned or unconditioned fear stimuli to elevate the startle response (Hitchcock & Davis, 1991). NMDA antagonists infused into the amygdala prevent the acquisition of fear-potentiated startle. These data indicate that an NMDA receptor-mediated process at the level of the amygdala may be critical for development of fear conditioning (Fendt, 2001).

Table 2-1: Animal models eliciting a conditioning fear response and their associated brain areas and effect on cognitive-affective processes (Uys *et al.*, 2003).

Disorder	Models used	Brain areas involved	Cognitive-affective process
Generalized anxiety disorder	Elevated plus maze	Poorly defined	General avoidance behaviours
Obsessive-compulsive disorder	Spontaneous stereotype, acrallick dermatitis and drug-induced stereotype	Corticostriatal circuits	Control repetitive movements
Panic disorder	Fear-potentiated startle	Amygdala, hippocampus, medial prefrontal cortex, dorsal periaqueductal grey	Fear conditioning
Social phobia	Primate hierarchy	Amygdala, corticostriatal circuitry	Social submission
Posttraumatic stress disorder	Rodent time-dependent sensitisation	Hippocampus, prefrontal cortex	Time-dependent sensitisation

2.2 Neurobiological models of stress

2.2.1 Fear conditioning

The mechanism of fear conditioning has often been suggested as a model for the re-experiencing phenomena in PTSD because of the association between traumatic recall and seemingly unrelated stimuli and the ensuing fearful response (Maren, 2001).

Exposure to a severe traumatic event results in fear conditioning that serves an adaptive purpose as long as the threat is present. After termination of danger, the fear response is normally reduced (extinguished and/or diminished due to a contextual change, for example soldiers returning from Iraq). The fear response however, may also persist despite termination of the threat, even after a person has been removed from the threatening context. When the fear response generalizes, it is evoked by stimuli only remotely related to the initial traumatic event and then reflects sensitisation and cross sensitisation of the neuronal system. It is at this stage that all the symptoms of PTSD are observed (re-experiencing; avoidance; hyperarousal). The emotional response is highly stressful and in severe cases patients with PTSD may even lose orientation to time and place. Hypothesized mechanisms that promote maladaptive fear conditioning are enhanced recall, deficit in integration of context and content and impaired alternative learning. Figure 2-1 represents a cascade of events that can be strengthened by enhancement of fear conditioning and by delayed escape behaviour that are both part of the learned helplessness syndrome (Bonne *et al.*, 2004).

Conditioned fear responses have been studied mostly in rodents (Bonne *et al.*, 2004) to better understand the mechanisms underlying particular cognitive-affective processes (Table 2-1). If a conditioned stimulus (CS) which is non-threatening (e.g. a signal/ reminder) is presented together with an aversive / unconditioned stimulus (US) (e.g. shock), an animal soon exhibits a fear response termed conditioned response (CR). A CR is also evoked when the animal is placed in the environment (e.g. cage) in which the experiment took place. These two aspects of fear conditioning are termed "explicit cue" and "context" conditioning. Fear conditioning may with a single exposure of both CS and US, be very rapidly acquired (Maren, 2001) to induce a conditioned response to previously neutral stimuli (LeDoux, 2000). The reduction in fear that follows extinction does not result from forgetting or memory

erasure (Pearce & Bouton, 2001), rather it involves the formation of new non-aversive associations that "compete" with the prior fear-conditioned associations. It is important to note that the latter is not erased, and may be reactivated by particular circumstances after extinction (Bouton, 2000).

Fear conditioning is a highly adaptive mechanism in life-threatening circumstances (Aardal-Eriksson *et al.,* 2001; Sanders *et al.*, 2003), optimizing response to hazards, ensuring vigilance to potential danger and preventing diverting of attention to meaningless stimuli (Grillon, 2002). The conditioned fear responses to predator smell in an animal clearly demonstrate survival benefit and this occurrence of fear conditioning phenomena across species supports an evolutionary role for this mechanism (Stork & Pape, 2002).

In contrast, conditioned responses in PTSD are maladaptive (refer to Figure 1-1) and bring about fear and apprehension. PTSD-patiens are impaired in their ability to discriminate between threat-related and non-related stimuli because traumatic events are re-experienced in response to diverse trauma related and non-trauma related, ill defined stimuli. Re-experiencing symptoms in PTSD result from implementation of impaired and maladaptive fear conditioning-like mechanisms in response to severe stress. Maladaptive acquisition of re-experiencing symptoms is enhanced by non-associative learning processes and is further facilitated by learned helplessness behaviour (Bonne *et al.,* 2004).

The following complementary mechanisms may explain the transition of controllable (adaptive) fear conditioning into uncontrollable (intrusive) re-experiencing in PTSD, and will be briefly discussed:

a). Emotional-fear memories are more vividly encoded and more amenable to recall.

Arousing, fearful or emotionally exciting events are remembered better and for longer periods of time than emotionally neutral events (McGaugh, 2000). Traumatic events stimulate the release of cortisol, CRF, E and NE and therefore it has been hypothesized that these neurotransmitters enhance consolidation of emotional memory (McGaugh *et al.,* 1996; Roozendaal, 1999). Increased peritraumatic hormonal or catecholaminergic secretion and/or increased sensitivity in vulnerable individuals may lead to augmented emotional memory storage or enhanced retrieval and evolution of PTSD (Bonne *et al.,* 2004). Re-experiencing accesses emotional

memory. Short and even fully consolidated long-term memories (LTM) become unstable upon reactivation (Myers & Davis, 2002). Therefore, reactivated memories require another round of consolidation to return to memory storage, a process referred to as reconsolidation (Nader *et al.*, 2000). Clearly, memory reactivation is very common in PTSD.

b). The unconditioned response (UR) is maintained even in the absence of an unconditioned stimulus.

In the absence of an US (threat), re-experiencing in PTSD due to a non-threatening stimulus, often evokes an emotional response that is very similar to the one evoked by an US. Repeated non-threatening stimuli can also trigger re-experiencing but this may be conceived as a process of "priming" whereby an augmented emotional response occurs after repeated provocation by the same stimuli (Quirk & Gehlert, 2003).

According to Eyesenck's theory (1968), in select cases where there is an overlap between a conditioned response and an unconditioned response, repeated presentation of the non-threatening stimuli (in the absence of the threatening stimuli) may permanently stabilise fear conditioning rather than promote extinction (Bonne *et al.*, 2004). The unconditioned response is brought about by emotional and mental representations of the traumatic event(s), triggered by a non-threatening stimulus. This capacity for vivid mental imagery and representation may be unique to humans, partly explaining resistance to extinction in PTSD (Mineka & Zinbarg, 1996).

c). The capacity to integrate context and content related information into one coherent stimulus is absent.

The correct identification of safe and unsafe stimuli depends on proper integration of content and context information and serves a highly adaptive purpose (Bonne *et al.*, 2004). Adaptive fear conditioning enables the individual to distinguish between safe and unsafe (threatening) stimuli and to respond accordingly. The intense fear response of patients with PTSD to non-threatening stimuli, regardless of context suggests impairment in their capacity to integrate context and content stimuli, illustrating how fear conditioning completely loses its adaptive function (Bonne *et al.*, 2004).

d). Conditioned response to generalized stimulus is not diminished.

Modification of the non-threatening stimulus has been shown in fear-conditioned animals to elicit a reduced fearful response (Brandon *et al.,* 2000). This process serves to maintain the adaptive capacity (specific recognition) of fear conditioning. In contrast, in PTSD modification of the non-threatening stimulus does not lead to a decrease in fearful response. A fear response is evoked by numerous, diverse stimuli, whose semblance to the traumatic event is not recognisable. Therefore, "generalization" of non-threatening stimuli in patients with PTSD may represent synergism between associative forms of learning (Bonne *et al.,* 2004).

e). Extinction is absent despite stimulus non-reinforcement.

Persistent PTSD symptoms may also be viewed as a failure to extinguish the CR. Extinction is a process of alternative learning, rather than one of forgetting; therefore the absence of CS reinforcement by an US will not in itself enable extinction unless accompanied by active learning. This capacity may be impaired in PTSD (Bonne *et al.,* 2004).

The concept of extinction as a competitive learning process rather than memory erasure is also compatible with PTSD phenomenology because the foremost vulnerability factor for acquisition of PTSD is past history of trauma and childhood abuse (Koenen *et al.,* 2002; Banyard *et al.,* 2001). This re-exposure to a traumatic event (threatening stimulus), even after extinction, could trigger PTSD. Such re-exposure could be particularly deleterious if occurring in a surrounding where the individual does not feel secure. Likewise, this suggested mechanism of extinction may help explain the occurrence of "delayed" PTSD, whereby patients who initially extinguished a posttraumatic fear response present with "delayed" PTSD upon exposure to a particular cue or context. In addition, even "asymptomatic" patients with (past) PTSD when faced with circumstances even remotely similar to their initial trauma, respond with an intense emotional reaction (Toren *et al.,* 2002; Solomon, 2001).

f). Fear conditioning is enhanced following exposure to inescapable stress and resultant learned helplessness behaviour.

If the response to stress fails to alleviate the danger and fear, the stress will then be perceived as "inescapable". This experience is often accompanied by a behavioural pattern analogous with the animal model of "learned helplessness". Fear conditioning is enhanced in this state, creating a vicious cycle of positive

reinforcement between fear, inescapable shock, learned helplessness and fear conditioning (Bonne *et al*, 2004).

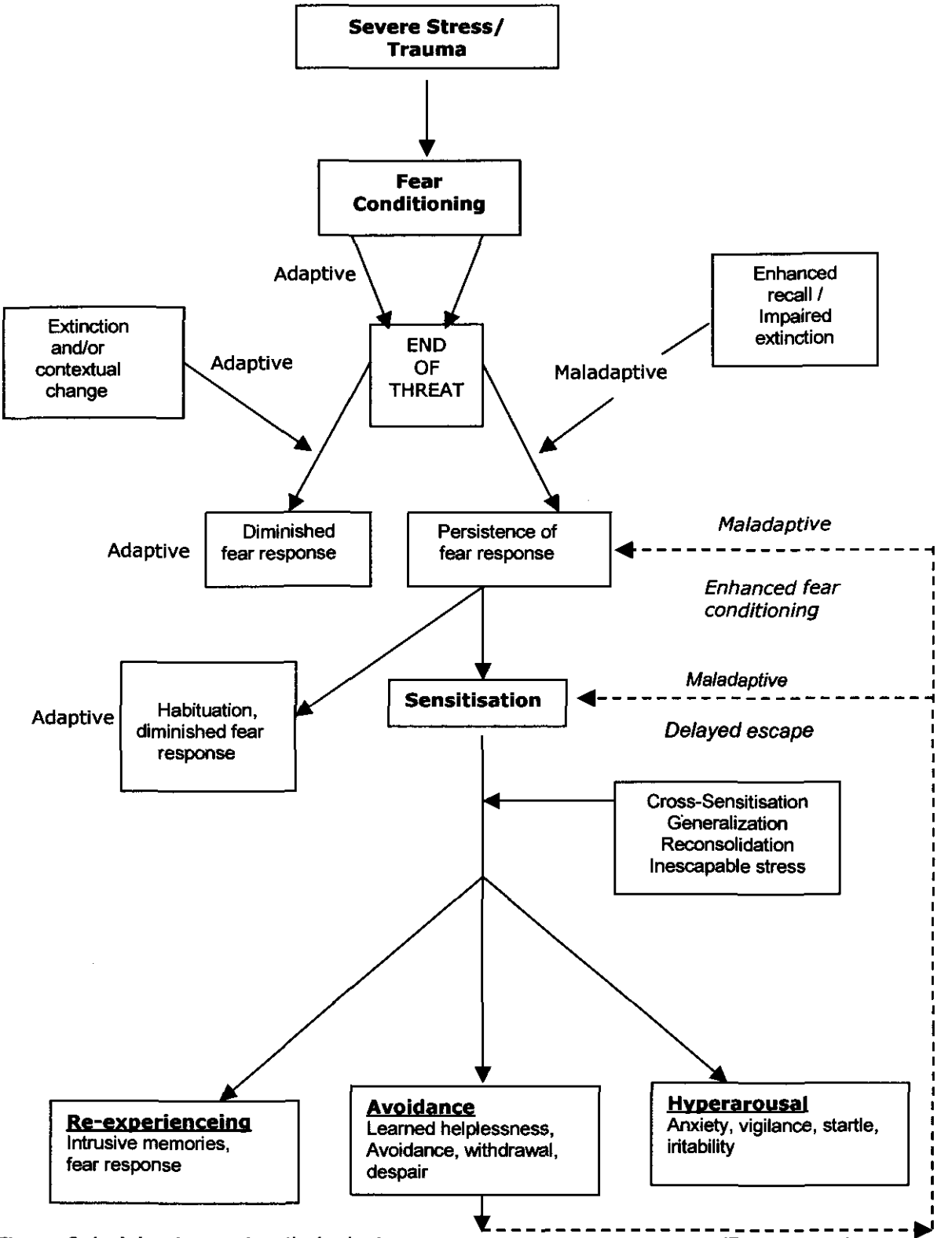


Figure 2-1: Adaptive and pathological responses to a severe stressor (Bonne *et al.*, 2004). Refer to text for a detailed description.

2.2.2 Learned helplessness (LH)

In this paradigm, an animal is initially exposed to uncontrollable (inescapable) stress. When the animal is later placed in a situation where shock is controllable (escapable), the animal not only fails to acquire the escape responses but also often makes no effort to escape the shock at all (Shirayama *et al.,* 2002). In humans, avoidance symptoms, both emotional (thoughts, feelings or conversations associated with the trauma) and physical (activities & people or places that may recall the event) as well as depression like symptoms (emotional numbing, loss of interest, social avoidance and withdrawal) are similar to the "learned helplessness" description of animal behaviour after exposure to severe stress (Maier, 2001).

The use of the acute learned helplessness model in PTSD remains controversial, whereas its appropriateness as an animal model of depression has been extensively validated (King *et al.,* 1993; Willner, 1991). Therefore, a genetic animal model of congenital learned helplessness (cLH) has been used to explore the role of genetic predisposition in PTSD (King *et al.,* 2001). The cLH model selectively utilizes an unpredictable uncontrollable stress event to determine individuals with an escape deficit in a subsequent controllable stress paradigm. Stress is often described as "inescapable", "unavoidable" and "uncontrollable" as well as "chronic" and "continuous" (Bonne *et al.,* 2004). All these terms denote exposure to significant stress, and convey the idea that learned helplessness is observed after major stress, presumably accompanied by severe subjective distress. The animal model of inescapable stress followed by learned helplessness has been used as an animal analoque of depression, anxiety and PTSD (Maier, 2001). This model is particularly suitable to describe the "avoidance" symptom cluster of PTSD.

The essential adaptive mechanism of fear conditioning becomes maladaptive in PTSD (refer to Figure 1-1), the conditioned stimulus becomes generalized, unpredictable and uncontrollable, and a person feels constantly threatened. This state is comparable to inescapable stress and results in learned helplessness behaviour (Chilcoat & Breslau, 1998). The standard measure of whether learned helplessness has been attained after stress exposure is "delayed escape" (Overmier & Seligman, 1967). It is assessed by determining the time required for animals to learn that a given behaviour will terminate shock / stress. Learned helplessness animals require a significantly longer time to learn this (Overmier & Seligman, 1967), to the extent that "delayed escape" has become perhaps the foremost criterion for

learned helplessness. Exposure to inescapable stress and the resultant learned helplessness has been shown to enhance fear conditioning (Maier, 1990; Shors *et al.*, 1992). The continuous re-experiencing and resultant distress in PTSD, together with emotional memory storage mechanisms such as reconsolidation (Myers & Davis, 2002), suggest that fear conditioning in PTSD is not a one time event but rather a continuous accumulative process. Delayed escape behaviour and facilitation of fear conditioning enhance this process even more, and can be conceived as providing "positive feedback" to the process of fear conditioning acquisition.

2.2.3 Kindling / Sensitisation

Animal studies indicate that the glutamate / DA pathways are responsible for inducing kindling (Post *et al.*, 1995). The kindling model rests on a delicate balance between inhibitory GABA-ergic and excitatory glutamatergic pathways and is a proposed model of bipolar type mood disorders (Davies *et al.*, 1991). However it has also been used to explain certain types of neural plasticity including learning, memory, addiction and anxiety. The GABA-ergic pathways play an essential, permissive role in the kindling action of glutamate (Davies *et al.*, 1991), whereas DA plays a significant role in tolerance and sensitisation mechanisms (Post *et al.*, 1995).

The kindling model was first described by Goddard *et al.* (1969) and is based on the observation that if sub-threshold stimulation is delivered on a timed basis the animal develops a progressive alteration in behaviour. If such sub-seizure threshold stimulus is continued, the animal develops a generalized convulsion with each stimulus. If continued even further the animal develops an independent seizure disorder unrelated to further stimulation (Clothier, 2004).

Work regarding the learned helplessness paradigm ties neuronal sensitisation to the behavioural impairment that follows unpredicted and inescapable stress (Minor & Hunter, 2002). The concepts of sensitisation and habituation are of particular relevance in the symptom cluster of PTSD. This cluster comprises symptoms of persistent anxiety and motor hyper-responsivity where patients lose their basic sense of safety (Janoff-Bulman, 1992). The adaptive function of fear conditioning to distinguish between safe and unsafe and facilitate identification of danger, fails in PTSD. This failure becomes manifest by the generalization of stimuli that can trigger the fear response. Repeated presentation of a stimulus may result in a progressively

decreasing response, termed "habituation", or a progressively increasing response, termed "sensitisation". Interestingly the same stimulus can cause habituation at one time and sensitisation at another. Both mechanisms are forms of non-associative learning, and may be prompted by environmental, behavioural, physical and neurochemical stimuli (Kandel & Schwartz, 1982). Repeated response to novel stimulus may result in an increased response to novel stimuli, a phenomenon termed "cross sensitisation" (Post *et al.*, 1999). Patients with PTSD are "sensitized" by their repeated fear response to recurring non-threatening stimuli. Once a person is "sensitised", even a non-threatening and neutral novel stimulus may evoke an excessive response.

2.3 Animal models of PTSD

2.3.1 Time-dependent sensitisation (TDS)

TDS refers to the fact that one exposure to a stressor (e.g. immobilization stress) can induce an extremely long lasting alteration in the subsequent responsiveness of the animal to pharmacological or non-pharmacological stressors. Therefore, the behavioural model of TDS has been proposed as a useful model for PTSD (Yehuda & Antelman, 1993). In this model, animals (e.g. rats) are exposed to a single session of prolonged stress (e.g. 2 hours of restraint followed by a 20 minute forced swim, followed by exposure to halothane or ether vapours). The animals are allowed to recover for a week, then they are subjected to a brief restress on day 7 (30 minute restraint stress or 20 minute forced swim stress). The rationale being that the frequency of exposure to situational reminders contributes to the maintenance of fear-related behavioural disturbances over time.

Animals subjected to TDS display enhanced sensitivity to negative glucocorticoid feedback that is characteristic of PTSD while also demonstrating distinct changes in mineralocorticoid and glucocorticoid receptor expression in the hippocampus (Liberzon *et al.*, 1999). Therefore, the TDS model has proved valuable for studying HPA abnormalities relevant to PTSD (Liberzon *et al.*, 1997; Liberzon *et al.*, 1999). Stress-restress also evokes significant spatial memory deficits together with lowered plasma corticosterone, which is consistent with clinical findings (Harvey *et al.*, 2003). Of particular relevance to treatment, is that TDS-stress evokes distinctive changes in hippocampal 5-HT_{1A} and prefrontal cortex 5-HT_{2A} receptors (Harvey *et al.*, 2003),

brain areas that are intimately involved in memory and stress responsiveness. The TDS model emphasizes the role of past trauma in predicting subsequent dysfunction, allows for the study of bidirectional expression of symptoms (enhanced or reduced responsiveness to environmental stimuli), and provides credible intrasubject variation (Yehuda & Antelman, 1993). In line with the increasing evidence for involvement of glutamatergic mechanisms in the pathology of stress and anxiety (Krystal *et al.*, 2002; Stewart & Reid, 2002), it is of interest that stress-restress evokes a significant increase in hippocampal nitric oxide synthase activity with marked changes in hippocampal NMDA receptors (Harvey *et al.*, 2004a).

2.3.2 Predator exposure

LH is a response unique to "inescapable" rather than "escapable" stress and is adaptive for animals in situations where both "fight" and "flight" response are ineffective, (i.e. when confronted by an "inescapable" predator) (Dixon, 1998). According to Adamec and Shallow (1993) there is a long-lasting increase in anxiety-like behaviour (ALB) in rats following inescapable exposure to a cat, although the rats could run from the cat (which is usually not aggressive and does not approach or investigate the rats). Furthermore, brief escapable exposure to a cat or cat odour increases defensive behaviours for many hours after threat removal. These reactions occur in rodents that had never been exposed to a cat, suggesting an innate recognition of the threat posed by the predator. It is these reactions to a recognizably life-threatening situation that may be particularly relevant to PTSD. Subsequent work have found evidence of dysregulation of the HPA-axis in rats exposed to cats and also elevated unconditioned startle responses with delayed habituation to startle stimuli (Adamec, 1997). Analogous changes in startle proneness are found in human PTSD sufferers (Adamec, 1997). Together, these findings suggest rodent response to predator stress has features in common with symptoms following traumatic stress in humans.

2.3.3 Electric shock

The acoustic startle paradigm consists of a ventilated, sound attenuated acoustic chamber. Acrylic animal holders are mounted on a response platform and any movement of the animal (e.g. rat or mouse) generates a digitized signal. Background

noise and acoustic startle stimuli are delivered by a speaker (Hebb *et al.*, 2003). The acoustic startle response (ASR) is a relatively simple response, characterized by rapid contraction of facial and skeletal muscles following an unanticipated and intense auditory stimulus. The startle response habituates over time following repeated presentations of the same stimuli (Koch & Schnitzler, 1997). Enhanced startle sensitisation following tailshock in rats causes non-habituation of the startle response and an increase of startle responsivity (Servatius *et al.*, 1995). This method has recently been proposed as an animal model of PTSD (Garrick *et al.*, 2001). Individuals with PTSD and other neuropsychiatric conditions with similar features of anxiety, display reduced habituation to startle presentations over time (Garrick *et al.*, 2001). In the rat, the appearance of increased startle in response to stressor-associated cues are correlated with the intensity of the initial stressor experience, including the frequency of shock infliction and number of stressor sessions employed (Servatius *et al.*, 1995).

In the classic model of LH, effects of daily sessions of brief electric shocks on avoidance-escape behaviour and open field activity last at most a few days (Weyers *et al.*, 1989). Longer lasting effects are found when much fewer shocks are concentrated in a short period of time. This model of long-term sensitisation after a short session of foot shocks has been extensively validated. It can cause altered reactivity in a wide range of novel challenges that can last for months, and is indicative of increased anxiety and startle responsivity (Servatius *et al.*, 1994).

Aggressive similar stressors, either single or repeated, cause social rejection that result in long-term changes in responsivity. Increased immobility reponses to strange stressors are similar to that seen in the foot shock model (Koolhaas *et al.*, 1990), however disturbances in temperature, circadian rhythms of heart rate and activity seem to be more specific for this model (Meerlo *et al.*, 1996). After social rejection, long-term disturbances become less pronounced when animals are housed in groups rather than being housed single (Ruis *et al.*, 1999). In contrast to the foot shock model, rejected rats show increased immobility in the forced swim test (Koolhaas *et al.*, 1990). Long-term sensitisation can also occur after social confrontations in the absence of physical wounding during the encounter. Rats that are forced to witness another rat being shocked show long-term changes in behavioural reactivity that have the opposite direction of that seen in the shocked rats, i.e. increased activity in a strange environment (Van den Berg *et al.*, 1998).

2.3.4 Underwater trauma

Underwater trauma in the Morris Water Maze (MWM) (Figure 2-2) is a method described by Morris (1984) to investigate spatial learning and memory in laboratory rodents. This model was developed as a rat model for PTSD in which the diagnosis will depend on behavioural and physiological symptoms that can be measured in rats (Richter-Levin, 1998). Underwater trauma is a brief exposure to a life-threatening situation, therefore it may represent a brief trauma (Yehuda & Antelmanm, 1993). The underwater trauma can be induced within context (in the maze) and out of context (in a different container and different room) and the effects of such trauma on memory and attention can be evaluated in the context of the trauma. According to Richter-Levin (1998), the underwater trauma may be a more natural setting than other types of stressors such as electrical tail shocks, and the MWM is easy to operate and is widely available.

The water maze consists of a circular pool (1.8 m in diameter an 0.6 m in height) filled with approximately 30cm of water. The pool is devided into four quadrants with the use of string, raised 30cm above the water level. For a spatial learning task a cylindrical platform (diameter: 8cm; height: 28cm) is hidden in one of the four quadrants with the top surface 2-3cm below the water level. In this way the platform is not visible to the performing rat. The task involves placing the rodent into the water where it must use visual cues to remember the location of the hidden platform. Probe trials are used to determine the rodent's ability to retrieve information learned in previous hidden platform tests. The path of the rat is recorded manually and the escape latency time is measured with a stopwatch.

During the underwater procedure, no platform is used in the maze. Rats have to swim for 1 minute and then they are held under water for another 30 seconds, using a special net. Thereafter the rats are put back in their cage until commencing the posttrauma test.

2.4 Stress paradigms

PTSD, which is a fairly circumscribed biobehavioural syndrome, can be induced by a wide range of stressors, whereas animal studies of stress have shown marked biobehavioural differences depending on the type of stressor studied (e.g. controllable, escapable, acute, chronic, predictable, physiological, psychological).

Second, differential responsivity to stress can be influenced by factors other than the actual stressor, such as the state of the organism during stress, past stress history of the organism, and even genetic makeup. Third, the stressor itself may be only one of many important variables contributing to the development of PTSD (Yehuda & Antelman, 1993).

According to Yehuda & Antelman (1993) stressors should meet at least some of the following criteria:

- Even relatively brief stressors should be able to induce biological and behavioural sequelae of PTSD;
- The stressor should be capable of producing the PTSD-like effects in a dose-dependent manner;
- The stressor should produce biological alterations that persist or increase in time;
- The stressor should induce biobehavioural alterations that have the potential for bi-directional expression;
- Individual differences in response to a stressor should be present either as a function of experience, genetics, or an interaction of the two.

Chapter 3 The NO-cGMP pathway

3.1 Introduction

There is overwhelming evidence that the glutamate/nitric oxide (NO)/soluble guanylyl cyclase (sGC) system is of primary importance in a variety of physiological and pathological processes of the brain. Most of our knowledge on this neurochemical pathway derives from *in vivo* and *ex vivo* studies but the recent improvement of micro dialysis techniques combined with extremely sensitive measurements of the amplified end-product cyclic guanosine 3', 5'-monophosphate (cGMP) has added new impetus into its authors and functional relevance in the brain (Fedele & Raiteri, 1999). This system is a major, well conserved, signalling transduction pathway implicated in a wide range of physiologic and pathophysiologic functions of the cardiovascular, respiratory, gastrointestinal, and nervous or immune systems (Vulliemoz, 1998). These actions are all mediated by the activation of sGC and the consequent increase in the concentration of GMP in target cells (Moncada & Higgs, 1993). Although NO mediates both intra- and intercellular communications, it becomes cytostatic or cytotoxic when formed for long periods or released at excessively high concentrations. The important association between glutamate, GABA and the NO-cGMP pathways may hold the key to why severe mood and anxiety disorders are associated with neuronal atrophy and possibly also neural damage and cell death (Villarreal *et al.*, 2002).

3.2 Nitric oxide (NO)

Furchgott and Zawadzki (1980) identified an endothelial-relaxing factor, which they coined EDRF, which was localized in the vascular endothelial cells. It took many years to recognize that EDRF was either NO or a labile nitroso precursor that is metabolized to NO in the cell. NO is a ubiquitous compound that can serve two functions. Krumenacker *et al.* (2004) have summarized the mechanism of NO as follows:

(1) cGMP-dependent, which involves the production of the second messenger, cGMP, following NO activation of sGC; and
(2) cGMP-independent, which are mediated primarily by reactive nitrogen species (e.g. N_2O_3) that are produced as a result of the interaction of NO with oxygen (O_2) or superoxide radicals ($O_2^{\bullet -}$).

Nitric oxide is an inorganic compound with a half-life of a few seconds under physiologic conditions (Vulliemoz, 1998). It is a labile, small-molecule transmitter substance that exists as a free radical gas, though in most biological situations NO is in solution (Snyder & Dawson, 2000). Bruhwyler *et al.* (1993) have reviewed the role of NO in the olfactory- and circadian system, pain perception, wakefulness, food and water intake. More recently, its role has expanded to a probable involvement in various neurological and psychiatric disorders, including depression, anxiety, sensitisation (Harvey, 1996), Alzheimer's, Parkinson's and Huntington's disease (Kuiper *et al.*, 1994; Dawson & Dawson, 1998) and seizure disorders (Dawson *et al.*, 1992). NO occurs especially in areas of the brain that are involved in long-term behaviour and memory. NO is also thought to play a role in the pathology of several inflammatory disease states such as arthritis, myocarditis, colitis, nephritis and other pathological conditions such as cancer, diabetes and neurodegenerative diseases (Hanafy *et al.*, 2001). Disturbances in vascular NO synthesis are important in the pathophysiology of various vascular disorders including hypertension, arteriosclerosis and heart failure (Mayer & Hemmens, 1997). Many of the mechanisms that have been proposed to explain the impaired endothelium-dependent relaxation in atherosclerosis involve a decrease in either endothelial-derived NO production / cofactors for NO synthesis or eNOS gene expression (Shimokawa, 1999). The endothelial L-arginine/NO pathway is tonically active in resistance vessels and provides a physiological vasodilator mechanism that influences the peripheral vascular tone and hence systemic blood pressure (Rang *et al.*, 1999). In the cardiovascular system, both NO and cGMP can decrease myocyte L-type Ca^{2+} current and Ca^{2+} concentration through the activation of cGMP-stimulated cAMP phosphodiesterases (Balligand & Cannon, 1997).

Furthermore, NO also occupies an important place in the immune system, especially as a defence against invading micro-organisms, but also cancer cells (Lowenstein *et al.*, 1994). The rise in O_2^- and NO_3^- in human serum during infection serves as strong evidence to support the role of NO in the human immune system. During

bacterial infection, iNOS in macrophages release large amounts of NO, which destroys the phagocytosed bacteria by combining with the FeS centres in the enzymes critical for bacterial survival and reproduction (Lowenstein *et al.*, 1994).

However, the roles of NO as a second messenger, are enormously complex and are tightly regulated processes (Krumenacker *et al.*, 2004). As mentioned earlier, NO can have damaging effects to cells under certain conditions. The protective or toxic effect of NO depends on its chemical fate, and on the rate and location of its production. In the endothelium and CNS, NO acts as a neurotransmitter or neuromodulator with the subsequent activation of GC. This enzyme catalyzes the reaction of guanosine tri-phosphate (GTP) in the target cell to yield cGMP (Marletta *et al.*, 1990). Furthermore, NO and its derivatives stimulate expression of important proteins and enzymes at levels of transcription and translation, and to activate or inhibit activity of other proteins and enzymes (Vanin, 1998).

Clearly, understanding the NO-cGMP pathway requires understanding of NO's role as a signalling agent, the alternative targets for NO, the alternative activators of sGC and, naturally, the targets for cGMP (Denninger & Marletta, 1999).

3.2.1 NO biosynthesis

Two components underlie the NO cycle, namely, the reaction catalysed by NOS and the nitrite-reductase reactions catalysed by electron-donor systems (Reutov & Sorokina, 1997).

3.2.1.1 Enzymatic

Nitric oxide is produced in large quantities during host defence and immunologic reactions. In the brain, NO is produced by neuronal or endothelial isoforms of NOS and calcium dependent flavoenzymes (Bredt & Snyder, 1990). These enzymes have also been called EDRF synthase or guanylyl cyclase-activating factor synthase (GAF) (Murad, 1994).

Because NO cannot be stored by conventional means nor inactivated after synaptic release, its biosynthesis constitutes the only means for regulating NO levels (Snyder & Dawson, 2000). Biosynthesis of NO involves a two step oxidation from the

terminal guanidine nitrogen of L-arginine to L-citrulline, with concomitant production of NO (Andrew & Mayer, 1999). NOS enzymes are central in the control of NO biosynthesis, and catalyses the β-nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reaction of L-arginine with O_2 to yield NO and the amino acid citrulline (Figure 3-1) (Dash, 2001).

NOS displays recognition sites for NADPH, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). Direct biochemical analysis shows FAD and FMN bound stoichiometrically to NOS (Bredt & Snyder, 1994; Marletta, 1993). NOS is homologous to cytochrome P_{450} reductase (CPR), an enzyme involved in detoxification processes, because it is the only other mammalian enzyme that possesses recognition sites for both FMN and FAD as well as NADPH (Snyder & Dawson, 2000). NOS also utilize tetrahydrobiopterin (BH_4) as an electron-transferring cofactor (Bredt & Snyder, 1994; Marletta, 1993). It is likely that the mechanism of electron transfer is similar to that of the P_{450} enzymes namely, that NADPH reduces FAD, which reduces FMN, which, in turn, transfers electrons to the ferric heme, promoting the interaction with molecular oxygen (O_2) (Snyder & Dawson, 2000). The exact role of BH_4 is not clear but probably involves a stabilization of the enzyme (Bredt & Snyder, 1994; Marletta, 1993).

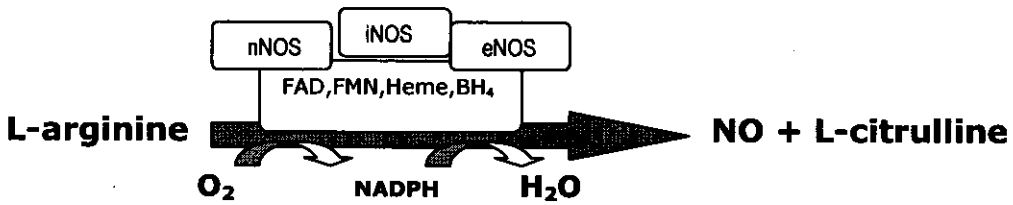


Figure 3-1: Schematic representation of the formation of nitric oxide and L-citrulline from L-arginine by NOS and several cofactors.

3.2.1.2 Non-enzymatic

Non-enzymatic generation of NO resulting from acidification and reduction of nitrite (NO_2^-) was shown in the ischaemic heart to be responsible for myocardial injury and loss of contractile function. This suggests that NO from dietary sources of NO_2^-/NO_3^- may participate in inflammatory reactions, independent of NOS activation (Eiserich *et al.*, 1998). The participation of haemoglobin in the blood and methaemoglobin in muscle tissue contributes to the conversion of NO_2^- to NO, by nitrite-reductase,

making NO_2^- part of the integral system which participates in NO synthesis. NO-synthases are not present in all mammalian cells. The presence of mitochondria and endoplasmic reticulum are important in this regard, whose electron transport chains can participate in reducing NO_2^- to NO. The rate of NO formation during the course of this reaction is at least 1000-fold higher than that of the NOS reaction (refer to Reutov & Sorokina, 1997 for review). However, exogenous exposure to NO can occur through air pollution from both cigarette smoke and automobile combustion gases (Eiserich *et al.*, 1998).

NO is known to participate in the regulation of intracellular concentration of calcium (Ca^{2+}) (Reutov & Sorokina, 1997). The first regulatory mechanism is related to NOS activation, an increase in intracellular concentration of cGMP, and the activation of Ca^{2+} -pumps of the endoplasmic reticulum through G-kinases (Garthwaite, 1991). The second mechanism involves the release of Ca^{2+} from its pool that is insensitive to inositol triphosphate (IP_3) but sensitive to Ca^{2+} and cyclic adenosine diphosphate-ribose (cADPR). Consistent with this, activation of ADP-ribosyl-transferase with the participation of NO may influence the mechanism of Ca^{2+} release and one of the intermediates of NO metabolism, NO^+, can affect the permeability of Ca^{2+} channels (Galione, 1992). These properties of NO make it possible to consider it a secondary messenger, such as cAMP, cGMP, Ca^{2+}, IP_3, diacylglycerol (DAG), cADPR and arachidonic acid (Moncada *et al.*, 1991).

3.2.2 NOS enzymes

The neuronal NOS (nNOS) isoform releases NO that is involved in neurotransmission in the CNS. Inducible NOS (iNOS) is found in many tissues and in macrophages, and when induced by cytokines, produces NO as part of the body's immune response. Endothelial NOS (eNOS) releases NO resulting in inhibition of platelet aggregation and vasodilation via activation of sGC in underlying vascular smooth muscle cells (Thatcher *et al.*, 2004). nNOS is ubiquitously distributed within the brain (Table 3-1), such as the PFC, hippocampal 5-HT cell bodies and the dorsal raphe of the midbrain (McLeod *et al.*, 2001).

Initial efforts to purify the NOS enzyme were unsuccessful because of a rapid loss of enzyme activity upon purification (Snyder & Dawson, 2000). The discovery that calmodulin (CaM) is required for NOS activity in the brain permitted a simple

purification of brain NOS to homogeneity (Bredt & Snyder, 1990). Activation of the transcription factor nuclear factor $\kappa\beta$ (NF$\kappa\beta$) has been shown to play an important role in iNOS induction by bacterial products as well as by cytokines (Xie *et al.*, 1994). Knowels and Moncada (1994) have concluded that the constitutive isoforms can also be stimulated by various stimuli, such as Ach, bradykinin, thrombin, excitatory amino acids and leukotrines. The unique features of NOS enzymes are illustrated in Table 3-1.

Table 3-1: Unique features of the nitric oxide synthase (NOS) isoforms in the mammalian brain.

Classification	Type I	Type II	Type III
Isoforms	Neuronal (nNOS), (bNOS), (NOS-1)	Inducible (iNOS), (NOS-2), (macNOS), (hepNOS)	Endothelial (eNOS), (NOS-3)
Regulation & activation	Ca^{2+}/calmodulin, kinases (protein kinase Akt), changes in gene transcription, growth factors, neurotrophins, oestrogen	Ca^{2+} -independent, nuclear factor $\kappa\beta$ (NF$\kappa\beta$), interferon-responsive element, inflammatory mediators	Ca^{2+}/calmodulin, kinases (calmodulin-dependent kinase), catecholamines, autocoids, platelet derived mediators, mechanical forces, allosteric modulation, oestrogen
Subcellular location	Cytosol	Cytosol	Soluble & membrane bound
Function	Cell signalling	Cytotoxic Cytostatic Cytoprotective	Cell signalling
Expression in tissue	Paraventricular & supraoptic neurones, granule cells, β-pancreatic cells, skeletal muscle & epithelia of the lung, stomach & uterus.	Vascular cells, endothelial cells, pyramidal cells of CA1 & CA3 region, granule cells, cardiac myocytes, smooth muscle cells, macrophages, glial cells.	CNS parenchymal cells, smooth muscle and endothelium of vascular wall
Brain regions	Cerebellum, cerebral cortex, hippocampus, hypothalamus	Hippocampus, Cerebellum, Olfactory bulb	Hippocampus, cerebellum, forebrain, striatum, spinal cord, brain stem

The three NOS isoforms share 50-60% homology in their amino-acid sequences and are encoded by three different genes. Each isoform combine two functionally complementary portions, a carboxyl reductase- and an amino-oxygenase terminal domain (Figure 3-2). The reductase domain has binding sites for NADPH and the

flavin cofactors FAD and FMN. The oxygenase site binds CaM, BH_4, arginine, and heme (McLeod *et al.*, 2001). BH_4 is essential for the coupling of NADPH-dependent oxygen (O_2) activation to NO synthesis. The CaM binding domain plays a key role in both the structure and function of NOS and stimulates the rate of electron transfer within the reductase domain (Andrew & Mayer, 1999). The NOS dimer formation occurs by an intermolecular contact in the calmodulin-binding domain (Figure 3-2) where the head of one monomer is associated with the tail of the other monomer resulting in a dimeric structure (Reutov & Sorokina, 1997).

Dependence on Ca^{2+} is a key distinguishing feature between the constitutive and inducible isoforms of NOS (McLeod *et al.*, 2001). The neuronal and endothelial forms are constitutive in that stimuli for NO formation do not typically result in new enzyme synthesis (Snyder & Dawson, 2000). Instead, a stimulus in the brain (such as glutamate) acting on NMDA receptors triggers Ca^{2+} influx which binds to calmodulin, thereby activating nNOS (Snyder & Dawson, 2000). In addition, NOS can be found freely in the cytosol or interacting with other proteins. Cytosolic NOS may be close to intracellular Ca^{2+} reservoirs that control its activation (McLeod *et al.*, 2001). eNOS and nNOS are therefore both activated by an elevation in intracellular Ca^{2+}, and this dependence on Ca^{2+} may be ultimately due to the presence of an auto-inhibitory sequence which is present in the FMN-binding region of the two Ca^{2+}-dependent isoforms but is not found in iNOS.

The inducible NOS of macrophages and nonmacrophage sources are not stimulated by Ca^{2+} (Snyder & Dawson, 2000). Surprisingly, iNOS enzymes possesses CaM recognition sites. Cho and colleagues (1992) have shown that CaM is very tightly bound to iNOS, with the binding unaffected by Ca^{2+}, whereas CaM cannot bind to nNOS unless Ca^{2+} is present. The fact that CaM binds so tightly to iNOS and that it can be considered an enzyme subunit, accounts for the resistance of iNOS to Ca^{2+} activation (Nathan, 1992).

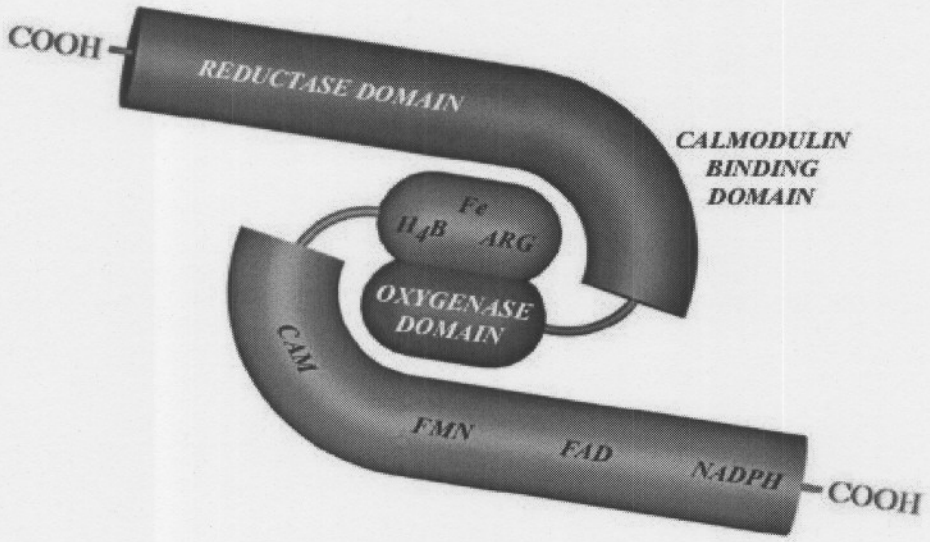


Figure 3-2: A schematic representation of the NOS enzyme (Dash, 2001).

3.2.3 Nitric oxide metabolism

The measurement of the stable end-products of NO metabolism, nitrite (NO_2^-) and nitrate (NO_3^-), are collectively known as nitrogen oxides (NO_x) and are commonly used as a rapid and simple way to assess NO production (Titheradge, 1998). NO is a colourless gas and is very stable when dissolved in water in the absence of O_2. However, in the air (at high concentrations) it rapidly reacts with O_2 to form nitrogen dioxide (NO_2) that is a brown gas capable of inducing tissue damage. In water and plasma NO is oxidized to NO_2^- which is stable for several hours, but in blood the nitrite is rapidly converted to nitrate (NO_3^-). Thus, NO_2^- basal concentrations in blood are low and those of NO_3^- are 100 times higher (Moncada & Higgs, 1993), but there is still little evidence for significant biological activity associated with NO_3^- (Thatcher *et al.*, 2004). Endogenous NO_3^- may be formed *in vivo*, for example in lipid peroxidation radical chain termination by NO (Nicolescu *et al.*, 2002). In contrast to NO_2^-, NO_3^- seems to act as exogenous NO sources/donors suggesting these compounds to be the more important exogenous therapeutic agents for application in illnesses other than angina and cardiovascular conditions (Thatcher *et al.*, 2004).

NO can also be rapidly oxidized to higher oxides of nitrogen resulting in nitrosate molecules containing sulfhydryl groups, such as glutathione, cysteine and albumin. Furthermore, NO interacts with other heme-containing proteins including myoglobin, the prosthetic group of sGC, and enzymes containing iron-sulphur centres (Ignarro, 1991).

3.2.4 Nitric oxide as a neuroregulator

NO is an important neuromodulator, regulating the release of primary neurotransmitters in the CNS, such as NE, 5-HT, DA, Ach, excitatory and inhibitory amino acids, histamine etc. (Prast & Philippu, 2001; Heiberg *et al.*, 2002). Although NO-producing cells are scarcely spread in many tissues, the NO released may influence neurons in a widely extended area (Prast & Philippu, 2001). Under *in vivo* conditions inhibitors of NO (refer to section 3.7) decrease Ach release in the basal forebrain (Prast & Philippu, 1992) and in the nucleus accumbens (Prast *et al.*, 1995), and as expected, NO donors (refer to section 3.7) enhance Ach release in both brain areas (Prast & Philippu, 1992; Prast *et al.*, 1995). Furthermore, NO potentiates the actions of released glutamate by inhibiting synaptic reuptake into the neuron (Pogun *et al.*, 1994). In the CNS NO also functions as a regulator of hypothalamic action. It is these important neuromodulatory actions that have created awareness of the putative role of NO in the regulation of mood, anxiety, motor activity, memory and endocrine function and hence in disorders such as depression, bipolar disorders (Karatinos *et al.*, 1995), panic disorder (Gordge, 1998), schizophrenia, anxiety- and stress disorders (Harvey, 1996).

In the periphery, together with vasoactive intestinal polypeptide (VIP), NO plays an important role as an inhibitory neurotransmitter in non-adrenergic non-cholinergic (NANC) smooth muscle responses throughout the gastrointestinal tract (Dick *et al.*, 2002). Furthermore, in NANC nerves that synthesize NO, the released NO modulates the arterial tone and in the corpus cavernosum NO also leads to smooth muscle relaxation, implicating NO in the treatment of erectile dysfunction (RDTC, 2003).

NO is different from other small-molecule transmitters in its mechanism of formation in the presynaptic terminal and in its action on the postsynaptic neuron. It is not preformed and stored in vesicles in the presynaptic terminal as are other

transmitters. Instead, it is synthesized almost instantly as needed and then diffuses out of the presynaptic terminals over a period of seconds rather than being released in vesicular packets (Guyton & Hall, 1996). It does not react with receptors but diffuses into the immediately adjacent postsynaptic neuron as well as into other nearby postsynaptic neurons (Snyder & Dawson, 2000). In place of reversible interactions with targets, NO forms covalent linkages to various targets which may be enzymes, such as GC or other protein or non-protein targets. Inactivation of NO presumably involves diffusion away from targets as well as covalent linkages to an assortment of small or large molecules such as superoxide and diverse proteins (Snyder & Dawson, 2000). In the postsynaptic neuron, it usually does not greatly alter the membrane potential but instead changes intracellular metabolic functions that modify neuronal excitability for seconds, minutes or perhaps even longer (Guyton & Hall, 1996). The latter has made NO an attractive candidate as the retrograde messenger involved in memory.

Because of NO's unique synthesis, release and action, it represents a completely novel class of neuronal messenger and might be classified as an atypical neurotransmitter (Snyder & Bredt, 1992).

3.2.5 NO interactions

NO is a fairly non-reactive molecule (that contains an unpaired electron) rather than a highly reactive free radical gas. Most molecules do not react with NO because their molecular orbitals are filled with two electrons. Therefore, NO will only react with other substances that have unpaired electrons e.g., metal complexes, O_2 or $O_2^{\bullet-}$, (Beckman & Koppenol, 1996), which lead to multiple different effects and downstream events depending on the localization and amount of NO and its substrates (Krumenacker *et al.*, 2004). Under normal biological conditions, NO concentrations are relatively low and most likely will interact with heme complexes in proteins, such as cGC, haemoglobin, cytochrome P_{450}, or protonphyrin-IX. NO can also interact with non-heme iron proteins, metals such as zinc, protein radicals, ribonucleotide reductases and cyclo-oxygenases (Hanafy *et al.*, 2001). When NO interacts with oxygen species (O_2 and $O_2^{\bullet-}$), nitrosylation and nitration can occur respectively and at higher levels of NO (during inflammation), these events usually occur more frequently (Beckman & Koppenol, 1996). For example, when NO interacts with O_2, electron acceptors or metals it produces NO^+ that, in turn, interacts

with thiols such as cysteine residues within protein (Gaston & Stamler, 1999). This can lead to *S*-nitrosylation of the thiol. Protein nitrosylation is a chemical reaction that has been demonstrated to affect the function of numerous proteins, including transcription factors and signalling molecules. The DNA binding activity of NF $\kappa\beta$, for instance, is inhibited when it is nitrosylated which will affect gene expression of iNOS (Klatt *et al.*, 1999). Under proper conditions when NO interacts with $O_2^{\bullet-}$, the strong oxidant peroxynitrite ($ONOO^-$) is formed (Padmaja & Huie, 1993). $ONOO^-$ can lead to tyrosine nitration, which is the addition of a NO_2 group to the phenol ring in tyrosine. Nitration of a tyrosine in proteins can also alter their function (Viner *et al.*, 1999).

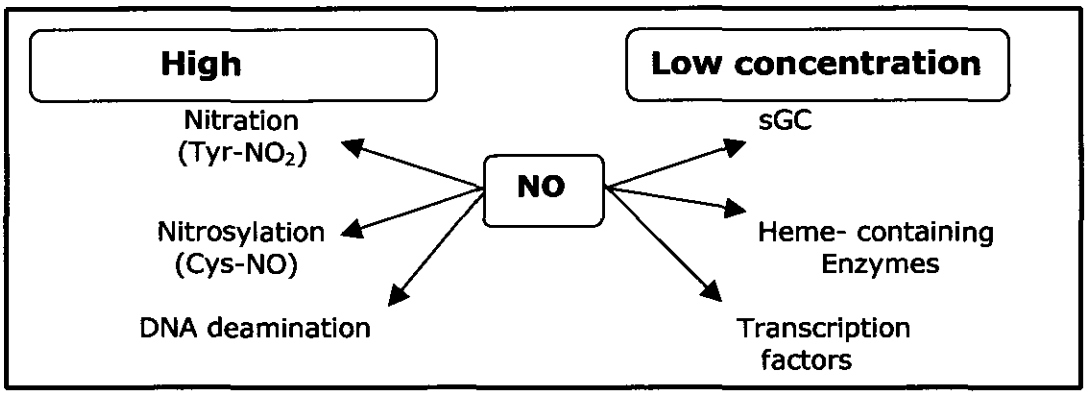


Figure 3-3: Effects of NO.

3.3 Cyclic guanosine 3′, 5′-monophosphate (cGMP)

3.3.1 Synthesis of cGMP

cGMP is synthesized by a family of enzymes referred to as the guanylyl cyclases (GC) (Figure 3-4), which share some amino acid sequence identity in the catalytic region to the adenylyl cyclases (AC) (Caccone *et al.*, 1999). Research has identified several isoforms of GC and each isoform can be classified dependent on its cellular location and each division represents structurally distinct proteins with specific properties (Hobbs & Ignarro, 1996). cGMP synthesis can be stimulated by various neurotransmitters such as glutamate (Barnstable *et al.*, 2004). In the CNS the synthesis of NO is linked to activation of the NMDA subtype of glutamate receptors

through the Ca^{2+} / calmodulin activation of NOS (Garthwaite, 1991). After glutamate has been released from the presynaptic terminal, NO diffuses out to neighbouring cells or to the presynaptic terminal(s) where it binds to the heme group of sGC resulting in the catalytic conversion of GTP to cGMP (refer to Figure 3-5) (Garthwaite, 1991). In some brain areas, there is a basal NO production which causes a tonic synthesis of cGMP (Vallebuona & Raiteri, 1993). The outflow of cGMP is greatly increased by activation of kainate/AMPA and NMDA receptors, while cGMP outflow is inhibited by NOS inhibitors and inhibitors of sGC (Luo *et al.*, 1994).

Barnstable and colleagues (2004) proposed a model whereby the activation of cyclic nucleotide-gated (CNG) cation channels in the cell membrane might regulate processes at synaptic terminals. They found that the conductance could be activated by application of NO donors, suggesting that the activity of the CNG channels might be coupled to NO signalling pathways. Consistent with this, they also observed that the conductance could be activated by application of cGMP-specific phosphodiesterase-5 (PDE-5) inhibitors (refer to section 3.3.2). This observation suggests that both cGMP production and hydrolysis in CNS neurons are continuous and that cGMP levels can be modulated by altering either synthesis or breakdown (Barnstable *et al.*, 2004).

3.3.2 cGMP metabolism

The duration of cyclic nucleotide signals within the cell are modulated mainly by their rate of degradation by cyclic nucleotide PDEs (Beavo, 2003). Pepicelli and collaborators (2004) concluded that basal cGMP is produced by the NO/cGC system in large amounts and is efficiently degraded by PDEs. Different kinds of cyclic nucleotide PDEs have been reported as regulators of both intracellular cAMP and cGMP (Kotera *et al.*, 2000). At present nearly 20 different PDE genes have been identified in mammalian species (Beavo, 2003), although only three or four different PDEs may be expressed by a particular cell type. Each cell type chooses its own PDEs to display the most appropriate and necessary functions (Beavo, 2003). PDE enzymes vary in their substrate specificity, some are non-selective between cGMP and cAMP (PDE-1, 2, 3, 10, 11), while others are selective for either cAMP (PDE- 4, 7, 8) or cGMP (PDE-5, 6, 9). PDE-5 is considered to be the main enzyme responsible for terminating the action af cGMP generated following the release of NO from nitrergic nerves (Gibson, 2000). Sildenafil acts as a potent and specific inhibitor

of PDE-5 (Davis & Waters, 1999), and effectively increases and prolongs the actions of endogenous substances that signal via the cGMP pathway (Gibson, 2000).

3.3.3 cGMP as a neuroregulator

Cyclic GMP is an established messenger molecule involved in numerous physiological processes (Koesling *et al.*, 2004). cAMP and cGMP have important functions as intracellular signalling molecules (second messengers) in the regulation of various cellular events in tissue. The physiological function of cGMP is the direct result of its interaction with several primary targets, such as cGMP-dependent protein kinases (PKG) which phosphorylates proteins, cGMP-regulated PDEs which mediate the activation or inhibition of cAMP catabolism, cGMP-regulated / cyclic nucleotide gated ion channels (CNG) and G-proteins (Goy, 1991). This signalling pathway mediates vascular and non-vascular smooth muscle relaxation and blood pressure regulation, cell growth and differentiation, inhibition of platelet aggregation, adhesion and secretion, bradycardia, and also neurotransmission, both peripherally and centrally (Prast & Philippu, 2001). Two genes encoding mammalian PKG exist and include PKG-1, which is highly expressed in vascular smooth muscle cells and PKG-2 which is most abundant in the brain and intestinal epithelium (Sellak *et al.*, 2002).

In the basal forebrain the release of Ach by NO donors is abolished by pre-superfusion with a GC inhibitor, LY-83,583 (Prast *et al.*, 1995), thus diminishing cGMP levels. In the nucleus accumbens cGMP mediates the NO-induced released of Ach by enhancing the outflow of glutamate. On the other hand, high concentrations of NO increase the output of GABA which in turn decrease Ach release (Prast *et al.*, 1998). Furthermore, the release of Ach in the nucleus accumbens elicited by NO donors is also abolished by LY-83,583 (Prast *et al.*, 1998). Microdialysis experiments have shown that the release of Ach in the striatum is stimulated by cGMP analogues (Prast & Philippu, 2001). Clearly, the NO-induced modulation of cholinergic neurons is mediated by cGMP.

cGMP also regulates the 5-HT transporter (SERT) (Miller & Hoffman, 1994), while sildenafil has been found to increase 5-HT transport in a dose-dependent mannner (Zhu *et al.*, 2004). Considering the important role for the SERT in antidepressant responses, the possible role of cGMP in regulating mood and anxiety, becomes more evident, especially with respect to drug response. According to Koesling *et al.*,

(2004), Ferrendelli and collaborators (1970) described an increase in cGMP levels in the cerebellum and cortex of mice and a concomitantly decrease in cAMP levels after treatment with Ach. Additionally, NOS-induced release of NO in the hippocampus also leads to the activation of cGMP-stimulated PDEs that is concentrated in these cells, thereby decreasing intracellular cAMP (Vincent *et al.*, 1998). Together with other observations, this led Goldberg (1973) to formulate his "ying-yang" hypothesis on the reciprocal effects of cGMP and cAMP. This reciprocal relationship has been described in the CNS after administration of psychoactive agents, such as lithium (Harvey *et al.*, 1990).

3.3.4 Guanylyl cyclases

3.3.4.1 Soluble guanylyl cyclase (sGC)

GC in the brain is distributed in a complementary way to nNOS (De Vente *et al.*, 1998a). sGC is a heme-containing cytosolic heterodimeric NO receptor (Krumenacker *et al.*, 2004) consisting of two subunits, α and β (exist in a 1:1 stoichiometry) (Figure 3-4) with the expression of both subunits being required for full catalytic activity (Buechler *et al.*, 1991). With respect to the NO-cGMP signal transduction pathway, the $\alpha_1\beta_1$-isoform of GC plays a pivotal role. This enzyme was originally purified from lung tissue and is found in the cytosolic fraction of nearly all mammalian cells. Two additional subunits, α_2 and β_2 are also present, with the α_2-subunit, but in most cases not the β_2-subunit, being able to form a catalytically active and highly NO-sensitive GC (Harteneck *et al.*, 1991).

Each sGC subunit contains three common domains that make up its structure and function, including the N-terminal heme-binding domain, dimerization domain and C-terminal catalytic domain, which is the most highly conserved region between the subunits, and is responsible for the conversion of GTP to cGMP (Koesling, 1999).

The soluble cytosolic forms are activated by various paramagnetic compounds originating from L-arginine and fatty acid hydrolysis and regulated by hemoproteins (Figure 3-4). The N-terminal region containing the heme-binding domain mediates the NO sensitivity of the enzyme (Ignarro *et al.*, 1982). Activation of NO-sensitive sGC is initiated when NO binds to the heme prosthetic group, which is a five-membered nitrogen-containing ring structure with a central ferrous iron. When NO

binds, the bond is broken between a histidine in the β_1 subunit (His_{105}) and the iron, forming a nitrosyl-heme complex (Stone & Marletta, 1994). Dimerization of both α_1 and β_1 subunits is required to obtain basal or NO-stimulated sGC activity (Buechler *et al.*, 1991) and in order for heme to bind properly to the heterdimeric complex both subunits are required (Foerster *et al.*, 1996). Dissociation of NO from the heme group triggers deactivation of GC (Koesling *et al.*, 2004). Mutations of Cys_{78} and Cys_{214} in the β_1 subunit of sGC reduce the affinity of heme for the enzyme, resulting in NO insensitivity. In contrast, however, mutations of the corresponding Cys_{145} and Cys_{284} in the α_1 subunit did not alter the sensitivity of the enzyme (Friebe *et al.*, 1997).

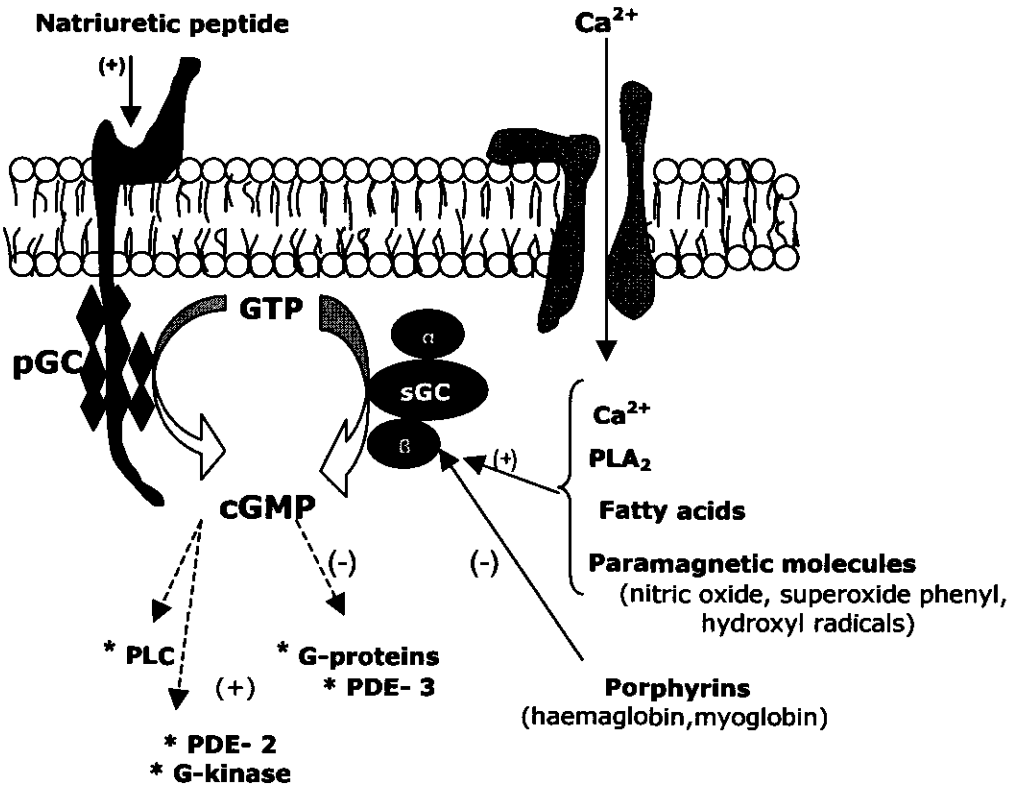


Figure 3-4: Biosynthesis and molecular sites of actions of cGMP (Harvey, 1998). PLC= proteinlipase C; PLA_2= proteinlipase A_2; pGC= particulate guanylyl cyclase.

3.3.4.2 Particulate guanylyl cyclase (pGC)

Particulate guanylyl cyclase (pGC) is a cell membrane receptor in various cells having a single transmembrane domain contiguous with intracellular GC. At least five distinct isoforms have been characterized. The primary role of pGC appears to be as a receptor site for the atrial natriuretic peptides (ANPs) (Figure 3-4) and an endogenous intestinal peptide, guanylin (Hobbs & Ignarro, 1996). There are three natriuretic peptide receptors (NPR) characterized, namely NPR-A; NPR-B and NPR-C. Both NPR-A and NPR-B are coupled to pGC which generates cGMP (Madhani *et al.*, 2003). NPR-C activates GTP-binding proteins that regulate AC and phosphoinositide turnover, suggesting a signal-transduction role for this receptor (Murthy *et al.*, 2000).

NO and natriuretic peptides function as vasodilators, via activation of the GC-cGMP pathway. Thus, NO activates the cytoplasmic heterodimeric haemoprotein sGC (Hobbs, 1997), while natriuretic peptides activate the membrane- bound pGC (Drewitt *et al.*, 1995). Stimulation of either cyclase results in the conversation of GTP to cGMP which is responsible for regulating cardiovascular homeostasis (Madhani *et al.*, 2003).

3.3.5 Actions of cGMP

In general cGMP effects are mediated via inhibition and stimulation of a number of PDEs which hydrolyze and inactive cAMP and via regulation of ion channels and activation of cGMP-dependent protein kinases (Pfeifer *et al.*, 1999). The result of these actions include changes in neuronal function, e.g. membrane ion flux, presynaptic changes such as Ach and NE release and uptake of 5-HT (Miller & Hoffman, 1994; Zhu *et al.*, 2004), promotion of neurite outgrowth and changes to various glial cell-directed events, e.g. hyperpolarization. NO-mediated cGMP synthesis also mediates induction of immediate early gene (IEG) expression (Haby *et al.*, 1994) which has been implicated, along with several other plasticity-related genes, in long-term synaptic changes and cellular memory (Arancio *et al.*, 1995). The combined actions of NO and / or cGMP predict that the pathway might have significant influence on neuronal function and in the regulation of psychic homeostasis and hence, in the pathophysiology of anxiety and mood disorders.

An important distinction, however, must be made between NO effects that are cGMP-dependent and others that are cGMP-independent (Sausbier *et al.*, 2000). The majority of NO effects on excitability depends on cGMP synthesis and the main signal transduction pathway of NO is activation of sGC, increase of cGMP formation and action of cGMP-dependent protein kinases (Smolenski *et al.*, 1998). Through the latter pathway, NO modulates the function of various cellular elements and ion channels (Prast & Philippu, 2001), reduces the function of AMPA receptors in the forebrain and cerebellum (McMahon & Ponomareva, 1996) and that of $GABA_A$ receptors in the cerebellum (Robello *et al.*, 1996). However, different cellular functions have also been found to mediate the main effect of NO, depending on the type of neuron and its location in the CNS. Modulation of receptors by NO and its toxic effects are some of the mechanisms relevant to the cGMP-independent way (Prast & Philippu, 2001). The latter mechanism of NO comprises direct reaction with proteins leading to nitrosylation and reaction of NO with superoxide resulting in the formation of $ONOO^{\bullet}$ and subsequent protein nitration and oxidation (refer to section 3.2.5). In cortical neurons NO enhances excitability by reducing GABA-mediated Cl^- influx independent of cGMP (Robello *et al.*, 1996). NO also enhances AMPA binding in the forebrain in a cGMP-independent manner by increasing the binding affinity (Dev & Morris, 1994).

3.4 The NO-cGMP pathway in the CNS

In the CNS, the NO-cGMP pathway is coupled especially to glutamatergic neurotransmission, triggering crucial events in synaptic plasticity involved in learning and memory, such as long-term potentiation (LTP) and long-term depression (LTD). Both LTP and LTD are associated with NO function (Boxall & Garthwaite, 1996).

The sequence of events from receptor activation to cGMP formation is well understood (Figure 3-5). Activation of either *N*-methyl-D-aspartate (NMDA) or muscarinic cholinergic receptors (excitatory receptors on the cell membrane), produce an increase in cytosolic calcium. In the presence of NADPH and several cofactors (refer to section 3.2.1.1) NOS is activated which catalyzes the oxidation of L-arginine to NO using molecular oxygen (refer to Figure 3-1) (Vulliemoz, 1998). The newly synthesized NO diffuses across the plasma membrane to neighboring cells where it activates sGC and increases the production of cGMP. It is of interest that little or no cGMP is formed in cells where NO is synthesized. This is because the

relatively high intracellular Ca^{2+} that activates NOS, inhibits GC activity (Vulliemoz, 1998). Furthermore, the Ca^{2+} also binds to CaM that activates PDE-5 which, in turn, inactivates cGMP to form 5'-GMP (Figure 3-5).

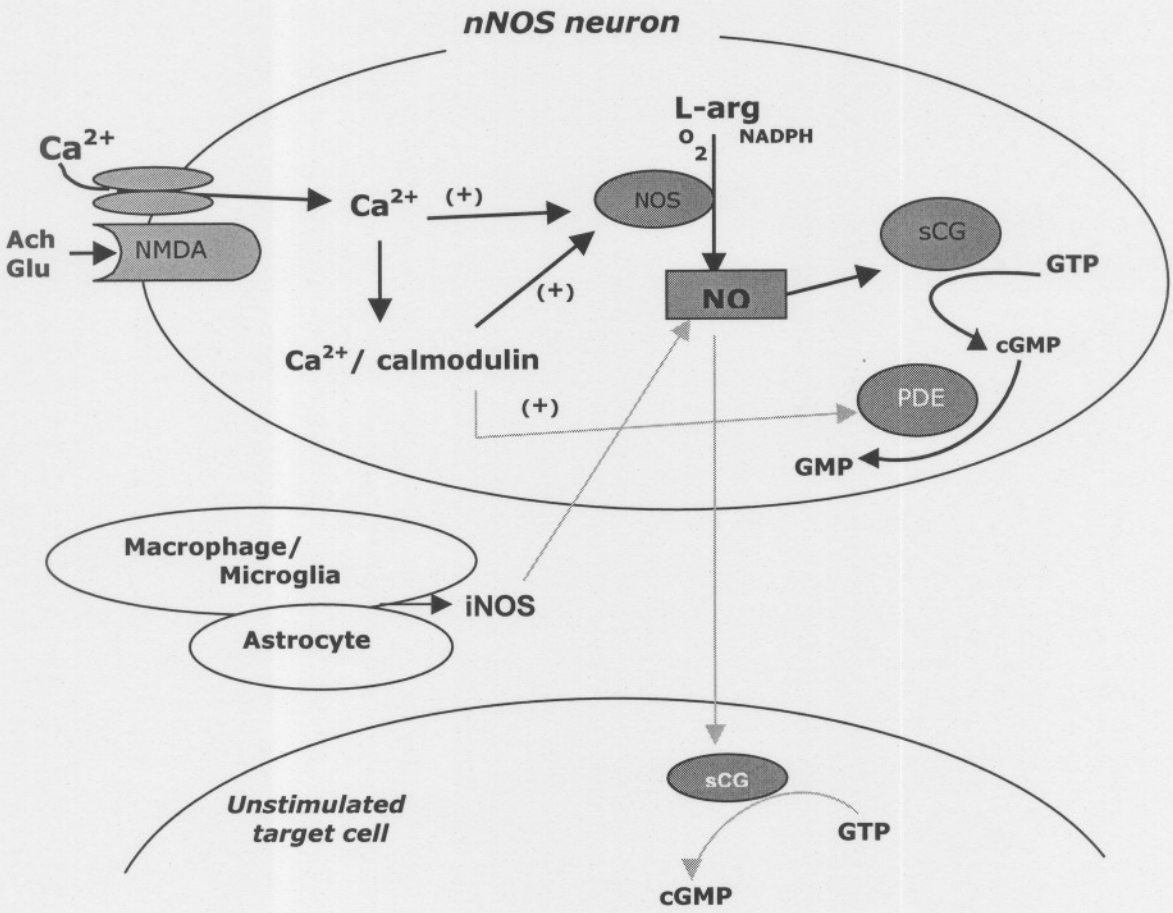


Figure 3-5: The NO-cGMP pathway (Vuliemoz, 1998).

NO is involved in a variety of physiopathological processes in the CNS (refer to section 3.2.5). Depending on its concentration, NO may elicit different effects (Figure 3-3), resulting from the interaction with sGC and the elevation of intracellular cGMP in target neurons, to other heme-containing proteins such as cytochromes, transcription factors etc. (Agulló, 2004). Pepicelli *et al.* (2004) have reviewed several intracerebral microdialysis studies of extracellular cGMP to investigate *in vivo* functioning and modulation of the NO-cGMP pathway, particularly in the cerebellum, cortex and hippocampus.

3.4.1 NO-cGMP signalling in the hippocampus

In the hippocampus, the NMDA-receptor/NOS/sGC-pathway triggers various forms of LTP (Pepicelli *et al.*, 2004), with NO synthase present in interneurons scattered within the hippocampus, dentate gyrus and neurons in the hippocampal formation. Microdialysis studies in the hippocampus confirm that NO donors (Table 3-2) elevate cGMP levels, whereas NOS inhibitors (Table 3-3) decreased extracellular cGMP. Moreover, application of NMDA via the microdialysis probe in the hippocampus produces a NOS-dependent increase in NO_x, as well as extracellular cGMP (Vallebuona & Raiteri, 1994), which can be prevented by NMDA receptor blockade, providing convincing evidence for the functional association between glutamate, NO and cGMP via activation of the NMDA receptor in this brain region.

NO plays an important role in LTP (Haley *et al.*, 1993), while inhibitors of sGC prevent the induction of LTP, suggesting cGMP involvement in LTP in the hippocampus (Lu *et al.*, 2002). Moreover, cGMP plays an important role in certain forms of memory (Prickaerts *et al.*, 2004). Clearly, the role of cGMP in LTP and memory needs serious consideration.

NO-cGMP signalling also plays as dominant a role in other brain regions, most notably the cerebellum and cerebral cortex, but are beyond the scope of the current study. Briefly in the cerebellum, LTP involves the concurrent stimulation of the climbing and parallel fibres resulting in diminished synaptic efficacy of the parallel fibre-Purkinje cell synapse (Dawson *et al.*, 1992). Since both LTP and LTD are associated with NO function, it has been postulated that this phenomena may underlie certain aspects of glutamate-induced cognitive and motor learning and memory (Rang *et al.*, 1999).

In the PFC, studies have indicated that extracellular cGMP levels are insensitive to NOS inhibitors (Laitinen *et al.*, 1994), suggesting that NO does not contribute to the production of the cGMP in the cortex, probably due to very high PDE activity. However, extracellular cGMP responds to various inhibitors of cGC, but only when PDE activity is inhibited (Pepicelli *et al.*, 2004). The PFC therefore seems to be the most efficient system for cGMP breakdown in comparison to the hippocampus and cerebellum (Vallebuona & Raiteri, 1994).

cGMP production in the rat frontal cortex involves activation of 5-HT_{2A} receptors (Regina *et al.*, 2003), although this increase in cGMP levels seems to be via an indirect cellular mechanism, involving the glutamatergic neurotransmission system (Regina *et al.*, 2004). Thus, the increase of NMDA-mediated cGMP production by 5-HT_{2A} receptor activation could involve an action directly on the NMDA receptor-ionophore complex, NOS, or sGC (Regina *et al.*, 2004). Furthermore, local administration of bicuculline, a $GABA_A$ receptor antagonist, augments cortical cGMP levels in the rat cortex, indicating that GABA, the major inhibitory neurotransmitter in the brain, also plays a key role in modulating the production of NO-cGMP (Pepicelli *et al.*, 2004). The NOS/cGMP system in the PFC is regulated by GABAergic, glutamatergic and serotonergic transmission.

3.5 NO-cGMP signalling and its role in PTSD

It is well known that increased glucocorticoid levels follow exposure to an acute stressor. However, PTSD develops and worsens over time and is often (Yehuda, 1997; Sautter *et al.*, 2003), but not invariably (Lemieux & Coe, 1995; Baker *et al.*, 1999) associated with a decrease in glucocorticoid levels. While further studies are needed to delineate the apparent paradoxical role of glucocorticoids in PTSD, various authors have put forward a primary role for glucocorticoids as well as glutamate in mediating the neurodegenerative pathology observed in PTSD, such as hippocampal shrinkage and cognitive impairment (refer to section 1.8.3.6).

Except for one small trial that investigated NO_x in acute PTSD (Yeh *et al.*, 2002), few studies have investigated the role of glutamate and NO in PTSD. Nevertheless, NO is a unique biological messenger that may promote the formation of free radicals and has been linked to neurodegenerative processes and psychiatric disorders, including PTSD (Ischiropoulos & Beckman, 2003). The local release of NO may modulate the release of stress hormones, for example CRF, ACTH and cortisol (Bugajski, 1999). Following activation of the stress axis (HPA-axis), glucocorticoids are thought to down-regulate the transcription and activity of NOS via a feedback mechanism (Lopez-Figueroa *et al.*, 1998).

Animal studies that emphasise repeated trauma, such as time-dependent-sensitisation (TDS), demonstrate an immediate elevation in hippocampal NOS activity that is sustained for a period of 3 weeks post stress (Harvey *et al.*, 2004a),

an elevation that appears to be driven primarily by iNOS. Excess production of NO can result in the formation of $ONOO^{\bullet}$ which can interact with polyunsaturated fatty acids resulting in lipid peroxidation, a well established mechanism of cellular injury (Lewen *et al.*, 2000). According to Homayoun *et al.* (2002) the iNOS isoform appears to play a more prominent role during chronic stress which can be experienced in the form of flashbacks and re-experiencing in patients suffering from PTSD and which is reproduced by TDS-stress. Inhibition of the TDS-induced activation of iNOS with the glucocorticoid synthesis inhibitor, KTCZ, also brings a strong association with stress-induced release of cortisol (Harvey *et al.*, 2004a). Since TDS-stress evokes biobehavioural changes similar to that seen in PTSD, such as decreased cortisol and memory deficits (Harvey *et al.*, 2003), the causal role of NO-cGMP warrants further investigation.

3.6 Measuring NO-cGMP activity in biological specimens

Because of the extremely short half life of NO in biological fluids, sophisticated techniques are needed to determine authentic NO, for example chemiluminescence detection, fluorescence detection; conversion of Fe(II)-oxyhaemoglobin (oxyHb) to methaemoglobin (metHb), and spin trap/ESR detection (using e.g., Fe(II)-oxyHb). Indirect methods include quantification of NO_2^-, or NO_3^-, using the Griess assay, measuring accumulation of cGMP, or determining NOS activity using radio-immunoassay. NOS protein can be determined using western blot analysis (Kuo *et al.*, 2003) or real-time polymerase chain reaction (RT-PCR) (Iemitsu *et al.*, 2000).

To improve sensitivity and reliability, combining some of the above mentioned assays may be more useful than using each assay on its own, eg. NOS assay, together with the Griess or cGMP assay. Furthermore, western blotting can be added to detect NOS protein expression together with RT-PCR that detects the expression levels of mRNA of NOS. Interestingly, Kleinbongard *et al.* (2003) has shown that the assay of NO_2^-, but not NO_3^-, provides a more reliable biomarker for endogenous NO production.

3.7 Pharmacology of NO and cGMP

3.7.1 NO

There is growing realization that NO_3^- may represent new therapeutic strategies in various cardiovascular and neurological applications (Wu *et al.*, 2004). This has been inspired by the application of NO_3^- in dementia, the association of NO_3^- and NO with regulation of glutamate receptors and the proposal of hybrid NO_3^- drugs containing the non-competitive, open-channel NMDA receptor antagonist memantine (Lipton, 2003).

Drugs that possess the ability to release NO have been called NO donors (De Belder *et al.*, 1995). Such compounds, together with their classification, are listed in Table 3-2. NO donors have been used traditionally as vasodilators (e.g., glyceryl trinitrate), in the treatment of thrombotic disorders and also to inhibit platelet aggregation at concentrations that do not affect blood pressure (e.g., S-nitrosoglutathione) (De Belder *et al.*, 1995). In gastrointestinal, genitourinary and respiratory disorders, NO donors may mimic nitrergic nerve-mediated responses and have been shown to be effective in the treatment of mal-functioning of sphincters in the gastrointestinal tract and impotence in diabetes (Moncada & Higgs, 1995).

Table 3-2: Nitric oxide donors (Feelisch & Stamler, 1996).

NO donors	Class	Abbreviation
Glyceryl trinitrate	Organic nitrate	GTN
Sodium nitroprusside	Inorganic iron complex	SNP
S-nitroso-L-cysteine	S-nitrosothiol	CysNO
S-nitrosoglutathione	S-nitrosothiol	GSNO
S-nitroso-N-acetyl-L-cysteine	S-nitrosothiol	N-ac-CysNO
S-nitroso-N-acetyl-DL-penicillamine	S-nitrosothiol	SNAP
3-morpholinosydnonimine	Sydnonimine	SIN-1

The NO-cGMP pathway can also be inhibited by several analogues of L-arginine. N^{ω}-monomethyl-L-arginine (L-NMMA), present in the human plasma and urine (Vallance *et al.*, 1992), and many other analogues of L-arginine (Table 3-3) are known to act as competitive inhibitors of both the constitutive and inducible NOS

isoforms (Moncada *et al.*, 1997), although their selectivity *in vitro* toward one or another NOS isoform may vary (Table 3-3).

Table 3-3: Inhibitors of NOS (Moncada *et al.*, 1997).

NOS inhibitor	Abbreviation	Inhibitory potency
N^{ω}-monomethyl-L-arginine	L-NMMA	nNOS = eNOS > iNOS
N^{ω}-nitro-L-arginine	L-NA	nNOS = eNOS >> iNOS
N^{ω}-amino-L-arginine	L-NAA	nNOS = iNOS > eNOS
N-$^{\delta}$ iminoethyl-L-ornithine	L-NIO	iNOS > eNOS = nNOS
7-Nitroindazole	7-NI	nNOS = eNOS = iNOS
7-Nitroindazole – monosodium salt	7-NINA	nNOS = eNOS = iNOS
Aminoguanidine		iNOS > eNOS = nNOS

Several clinical conditions may benefit from NOS inhibition or a decreased NO production, including inhibiting constitutive NOS, for example in conditions such as cerebral ischemia or epilepsy where overproduction of NO may lead to neurotoxicity, or by inhibiting iNOS in chronic inflammatory diseases, injury or septic shock (Griffith & Gross, 1996). Other NOS inhibitors include N^{ω}-nitro-L-arginine-methyl ester (L-NAME), N^{ω}-N^{ω} dimethyl-L-arginine (L-ADMA), N^{ω}-$N^{\omega'}$-dimethyl-L-arginine (L-SDMA), L-canavanine and methylene blue (MB).

3.7.2 cGMP

cGMP levels can be augmented using cGMP analogues or blocking cGMP hydrolysis by PDE-V (Pepicelli *et al.*, 2004). Sp-8-Br-PET-cGMP is another potent non-hydrolysable cGMP analogue that is an agonist of PKG, whereas Rp-8-Br-cGMP is an inhibitor of PKG (Barnstable *et al.*, 2004). Selective inhibitors of PDE-V include sildenafil, zaprinast, UK-122764 (Turko *et al.*, 1999) and vardenafil (Corbin *et al.*, 2004).

Several substances have been found to inhibit sGC, including the 1H-[1,2,4]oxidiazolo [4,3,-a]quinoxaline-1-one (ODQ) and NS2028, both potent and selective inhibitors of sGC in brain slices (Garthwaite *et al.*, 1995; Olesen *et al.*, 1998). Other inhibitors such as MB and 6-anilino-5.8-quinoline-quinone (LY-83583) show less specificity than ODQ (Mayer *et al.*, 1993).

3.7.3 Other mechanisms of modulation

Dexamethasone (DXM) is a potent inhibitor of iNOS gene transcription (Kleinert *et al.*, 1996). Regulation of iNOS gene transcription,via the nuclear factor $\kappa\beta$ (NF$\kappa\beta$) can be positively modulated by cytokines, such as interleukin-1 (IL-1) and TNF (Brigelius-Flohé *et al.*, 1997; O'Neill & Kaltschmidt, 1997). Conversely, NF$\kappa\beta$ can be inhibited by dithiocarbamates eg. PDTC, resulting in attenuated iNOS gene transcription (Milligan *et al.*, 1996). Glutamate agonists are also able to induce NF$\kappa\beta$, while glutamate antagonists are able to inhibit the activity of NF$\kappa\beta$ (Guerrini *et al.*, 1995). Other inhibitors of NF$\kappa\beta$ include DHEA, cyclosporin A and 3,4-dichloroisocoumarin (DCIC) (Gilmore, 2004).

Constitutive NOS also lends itself to modulation by external receptors, especially those regulating Ca^{2+} flux. Voltage-sensitive ion channels (VSC) including sodium (Na^+), Ca^{2+} and potassium (K^+) are clinically relevant as targets of pharmacological intervention (Shafer & Meyer, 2004). VSC include the high-voltage-activated (HVA) channels which require strong membrane depolarisations and the low-voltage-activated (LVA) channels which are activated by small depolarisations (Ertel *et al.*, 2000). The HVA channels consist of currents mediated by L- and N-type channels (Shafer & Meyer, 2004).

While clinically important therapeutic compounds acting on the L-type VSCCs are widely utilized to treat cardiovascular disorders including hypertension, myocardial ischemia and arrhythmias (Robertson & Robertson, 1996), N-type VSCCs have the potential for clinical application in neurological disorders characterised by excess NMDA-receptor-NO activity. Memantine is a well tolerated, uncompetitive NMDA receptor antagonist with strong voltage-dependent and rapid blocking/unblocking kinetics (Parsons *et al.*, 1999). Neuroprotective activity of memantine in models of chronic neurodegenerative diseases is seen at doses producing plasma levels within the therapeutic range (eg. 5mg/kg/d) and lacking negative effects typically observed with another NMDA receptor antagonist, viz. MK-801 (Parsons *et al.*, 1999). Thus, the difference between memantine and other NMDA receptor antagonists is not qualitative, but rather quantitative (refer to Parsons *et al.*, 1999).

These pharmacological targets may hold great promise in treating neuropsychiatric disorders characterised by excess glutamate-NO-cGMP activity. In this particular

study, a multi-directional pharmacological approach to treating stress-evoked responses will be attempted using drugs selective for various cites of the NMDA-NO-cGMP pathway, including the glutamate-NMDA receptor (memantine), nNOS (7-NINA), cGMP-PDE (sildenafil) and NF$\kappa\beta$ (PDTC). Figure 3-6 illustrates the latter pathway, indicating the targets for pharmacological challenge (orange boxes) addressed in this study.

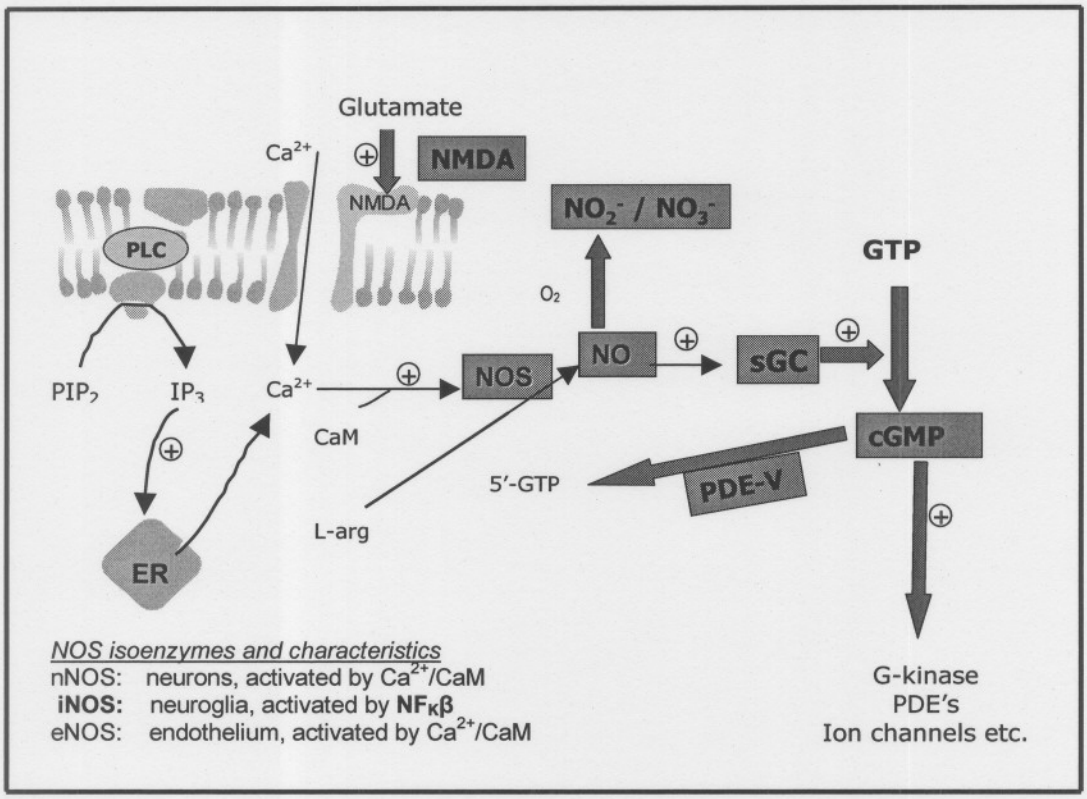


Figure 3-6: The pharmacological drug targets in the NO-cGMP pathway, used in the study. Refer to text for detail and additional abbreviations. ER= endoplasmic reticulum.

Chapter 4 Materials and Methods

4.1 Introduction

4.1.1 Rationale of the study

The NO-cGMP pathway holds great interest because of its apparent role in pathways involved in the response of the brain to severe stress. Thus, increased hippocampal expression of neuronal NO synthase (nNOS) and inducible NO synthase (iNOS) have been demonstrated to follow restraint stress in rats (De Oliveira *et al.*, 2000; Madrigal *et al.*, 2001), while iNOS has also been found to underlie the responses to TDS-stress (Harvey *et al.*, 2004a). It has also recently been found that selective serotonin reuptake inhibitors (SSRI's), which are the drugs of choice in the treatment of PTSD, inhibit NO synthase in the hippocampus (Wegener *et al.*, 2003). This provokes the hypothesis that altered NO activity may be an important neurobiological target in the pathology and pharmacology of PTSD.

NO, a second messenger activated by NMDA-dependent glutamatergic systems in various limbic and other brain regions (Kiss & Vizi, 2001), has been implicated in the neurobiology of anxiety and affective disorders (Kent *et al.*, 2002; Dager *et al.*, 2004). In support of this, pre-clinical studies have demonstrated the modulatory role that NO exerts on stress-induced behaviour (Masood *et al.*, 2003), while preliminary evidence suggests a role of NMDA-NO in the pathology of depression (Rosa *et al.*, 2003) and PTSD (Pall, 2003). The main target for NO seems to be soluble guanylyl cyclase (sCG), and a resulting increase in cGMP concentrations (Vallance & Collier, 1994).

A deeper understanding of the underlying neurobiology of PTSD is needed to enable the development of improved pharmacotherapy. The current study investigated the role of NO in stress, as well as the value of pharmacological manipulation of glutamate, NO and cGMP after exposure to the TDS-model, a putative animal model of PTSD (Uys *et al.*, 2003).

Because of the important role of the hippocampus in the stress response (Carrasco & Van de Kar, 2003) and that stress is known to adversely affect the hippocampus, this brain region was selected as the focus of this study.

4.1.2 Study objectives

The primary aims of this project were to:

- Determine the effect of TDS-stress in rodents on hippocampal NOS activity and accumulation of NO_x and cGMP.

- Include a pharmacological analysis of the model through sub-chronic administration of select agents active at various sites in the NO-cGMP cascade, viz. the NF$\kappa\beta$ antagonist, PDTC, the selective nNOS inhibitor, 7-NINA, the NMDA-receptor antagonist, memantine and the selective PDE-5 inhibitor, sildenafil, and to determine the effect of treatment on hippocampal NO_x as well as cGMP levels after TDS exposure.

- Determine the effect of each of the above-mentioned drugs alone, on the NO-cGMP cascade.

Since PTSD develops over time post-trauma (Yehuda & Antelman,1993), with prominent behavioural and neurochemical changes occuring on day 7 post TDS-stress (ps) (Harvey *et al.*, 2003; Khan & Liberzon, 2003), the current study looks at NO-cGMP changes on day 7 ps, while the effect of drug treatment is also evaluated after treatment, between day 1 ps and day 7 ps (Figure 4-2).

4.1.3 Study outline

The study consists of two groups of rats (n=10/group). Group 2 was exposed to the TDS paradigm as described in section 4.4, with group 1 serving as the control (Figure 4-1).

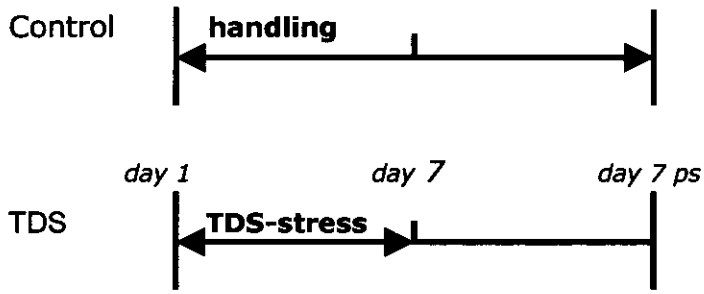


Figure 4-1: TDS-stress protocol.

Two sub-chronic pharmacological studies were set up, namely: (1) Control, TDS, and TDS plus drug treatment groups and (2) Control and separate sub-chronic drug treatment groups. During the first study, 4 animal groups (n=10/group) were exposed to the TDS–stress. After the re-stress period on the seventh day, the relevant drug treatments were given intra peritoneal (i.p). for another 7 days (Figure 4-2).

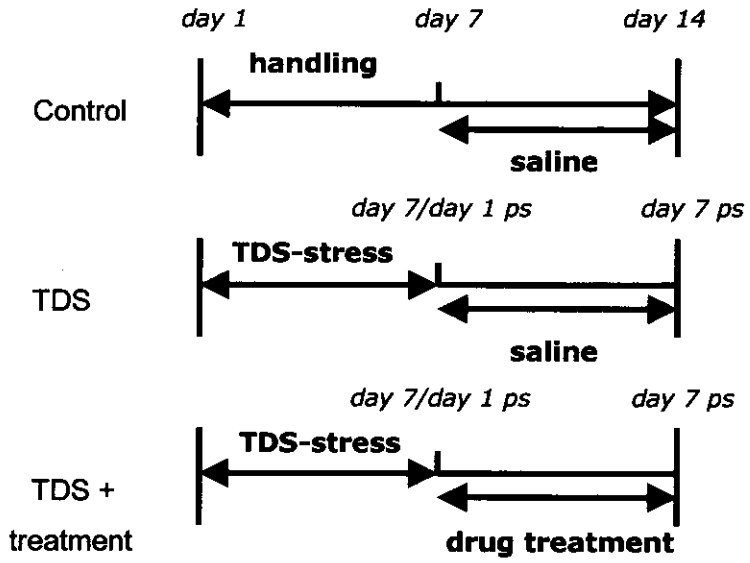


Figure 4-2: Drug treatment protocol for the first pharmacological study; effect of drug treatment on the TDS response.

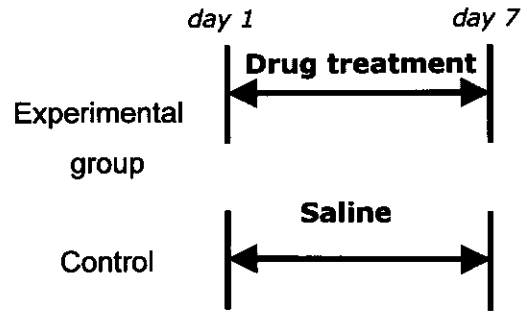


Figure 4-3: Drug treatment protocol for the second pharmacological study; effect of drug treatment alone in absence of stress.

In the second study, animals were treated with the same four drugs or saline separately for a period of seven days, without exposure to TDS-stress, to determine the effect of the drugs on their own (Figure 4-3). In all cases the rats had similar ages and weight. On day 7 ps, the animals were sacrificed, brains harvested and the hippocampi collected for assay of NOx and cGMP levels. Animals used in the control groups received either handling and/or i.p. saline injections (0.9% NaCl) as indicated in Table 4-1, thus exposing them to the same type of handling stress as the groups of animals receiving drug treatment.

4.2 Animals

The study protocol was approved and done in accordance with the guidelines stipulated by the Ethics Committee (approval number: 01D08) for the use of experimental animals at North-West University (Potchefstroom campus). Male Sprague-Dawley rats, weighing 200-250g, were used throughout the study and were provided by the Animal Research Centre of North-West University. The rats were housed in identical cages (six rats per cage) with a width of 28 cm, a length of 44,5 cm and a height of 12,5 cm (Figure 4-5). The rats were kept under constant conditions of temperature ($21 \pm 5°C$), relative humidity ($50 \pm 5\%$) and on a natural 12 hour light/dark cycle with free access to food and water. Full spectrum cold white light, with a light intensity of 350-400 lux was provided over a 12 hour light – 12 hour dark cycle. A positive air pressure was maintained in the facility with air filtration 99,7% effective for a particle size of 2 micron and 99,9% for a particle size of 5 micron. Rats received standard rat pellets with the following composition: protein

(180g/kg), fat (25g/kg), fibre (60g/kg), calcium (18g/kg), phosphor (7g/kg) and moisture (120g/kg). All animals were maintained according to a code of ethics in research, training, diagnosis and testing of drugs in South Africa.

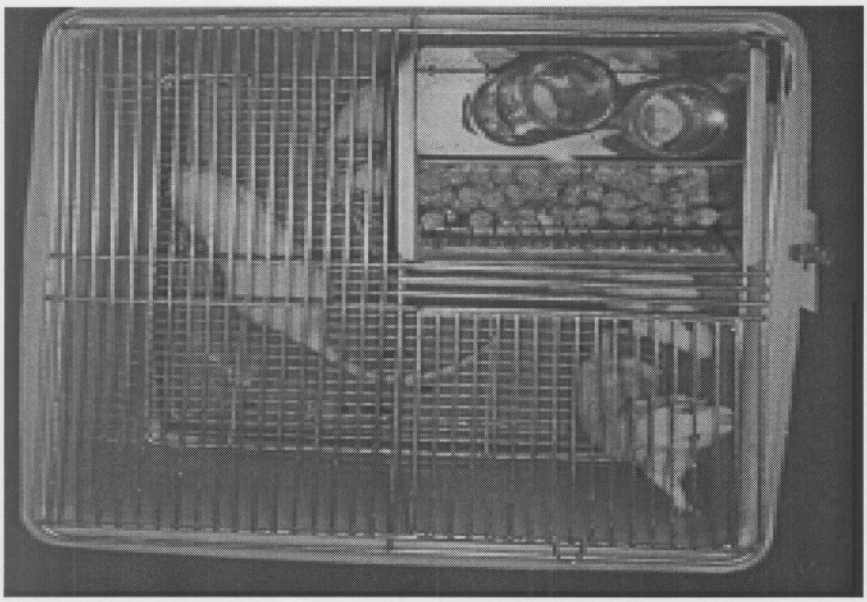


Figure 4-4: An example of the cages in which the rats were housed.

4.3 Drugs and chemicals

Drugs were chosen for their ability to selectively modulate the NOS/cGMP pathway, by either increasing or decreasing the activity of NO, cGMP, or both, in the brain of the experimental animals. All reagents used for the various assays were of the purest grade commercially available.

The following drugs were evaluated with respect to their effect on NO_x ($NO_2^- + NO_3^-$) production and cGMP levels in the hippocampus:

- Pyrollidine dithiocarbamate (PDTC), a NF$\kappa\beta$ antagonist, was purchased from Sigma-Aldrich, South Africa.
- 7-Nitroindazole monosodium salt, a nNOS inhibitor, was purchased from Tocris UK.
- Memantine, a NMDA receptor antagonist, was purchased from Sigma-Aldrich, South Africa.
- Sildenafil, a selective PDE-5 inhibitor, was kindly donated by Pfizer, South Africa.

4.4 The time-dependent sensitisation (TDS) model

4.4.1 Introduction

TDS has the potential to demonstrate the long-term effects of acute stressors in rodents (Antelman *et al.*, 1988). Previous studies have suggested a pathophysiological relationship between HPA-axis functioning and PTSD (Kanter *et al.*, 2001; Liberzon *et al.*, 1997). The TDS model is based on the principle of enhanced negative feedback, a quality of PTSD, and can be seen as a sensitisation of the inhibitory elements of the HPA-axis (Liberzon *et al.*, 1997). Furthermore, the model is also sensitive to the effects of single prolonged stress on plasma ACTH, corticosterone responses and glucocorticoid feedback (Liberzon *et al.*, 1997). These HPA-axis changes, together with bio-behavioural changes, akin to that seen in PTSD, has been observed after TDS-stress (Harvey *et al.*, 2003), and is sensitive to drugs that modulate 5-HT, a neurotransmitter known to play an important role in the treatment of PTSD (Harvey *et al.*, 2004b).

4.4.2 Description

TDS involves exposure of the animal to a severely stressful event followed by a situational reminder of the prior stress. The rationale being that the frequency of exposure to situational reminders contributes to the maintenance over time of fear-related behavioural disturbances and to the chronicity of the stress experience. This model has been fully validated and characterized in a previous project (Naciti, 2002) and has also been approved by the ethic committee (approval number: 01D08). The method follows a modified version of that originally described by Liberzon *et al.* (1997), but implemented using a slightly modified procedure described in Harvey *et al.* (2003).

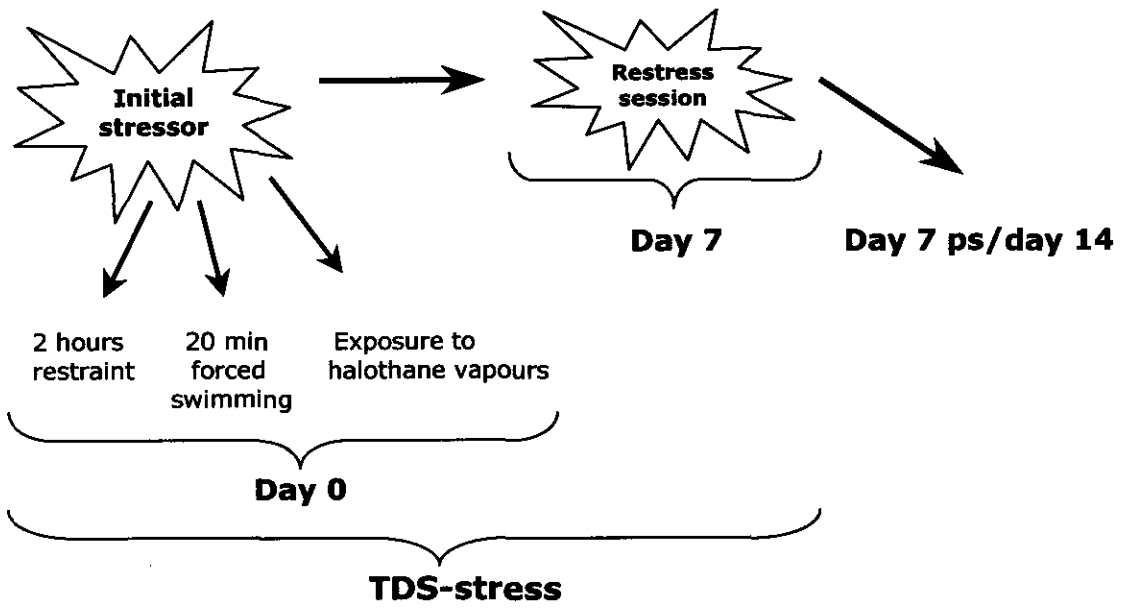


Figure 4-5: Schematic representation of the TDS model as described in sections 4.4.2 and 4.4.3. Between days 0 & 7 the animals were left undisturbed. Drug administration (one week) started on day 7 of the TDS period up to day 7-post stress (day 14) when the animals were sacrificed.

4.4.3 Methodology

The TDS procedure involves the exposure of the animal (rat) to sequential stressors that immediately follow one another (repeated stressors). This procedure includes a single session of prolonged stress on the first day, consisting of a two-hour restraint in a restrainer (Figure 4-6) followed immediately by a 20 minute forced swim (Figure 4-7). Thereafter, the animal is exposed to halothane vapours until loss of consciousness. The animal is then returned to its cage and left undisturbed for six days. On the seventh day a brief re-stress session is performed by exposing the rat to a single component of the initial-stressor performed on day 1, in this case, an additional 20 minute forced swim.

4.4.3.1 Stress procedure

4.4.3.1.1 Restraint Stress

Each rat is placed in a Perspex® restrainer (Figure 4-6) for two hours with the tailgate adjusted to keep the rat well contained without impairing circulation to the limbs.

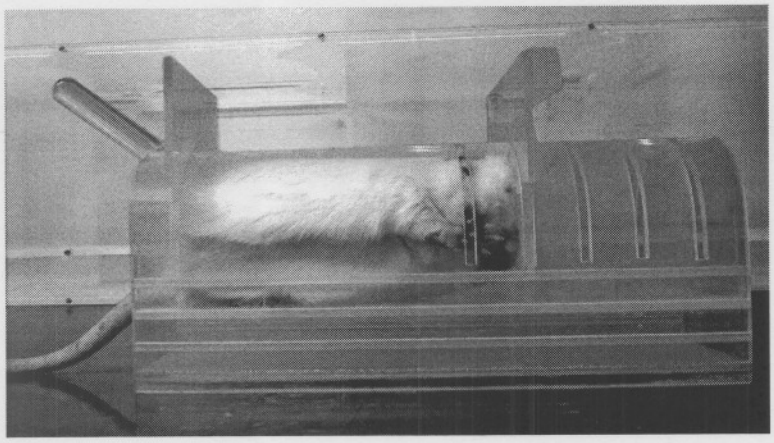


Figure 4-6: A rat exposed to the first stress session of the TDS model: restraint stress for 2 hours.

4.4.3.1.2 Forced swimming stress

After 2 hours of restraint stress, each rat is then individually placed into a Porsolt swim tank (Figure 4-7), filled with 25 cm of ambient water at 25°C. The depth of the water is adjusted to allow the animal to keep its nose above the water by using its tail as support. The animal is closely watched and the risk of drowning is zero.

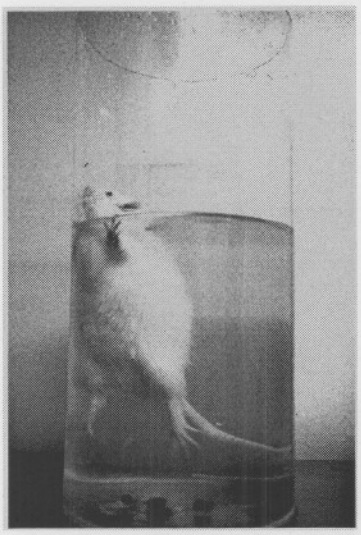


Figure 4-7: The same rat immediately exposed to the second stressor of the TDS model: 20 minutes forced swimming.

4.4.3.1.3 Halothane vapours

After 20 minutes of forced swimming, the rat is removed and immediately exposed to the 0.5ml of 4% halothane vapours in a 5ℓ sealed plastic container until loss of consciousness. Halothane vapours are very volatile and evaporated very easily, therefore an additionally 0.2ml is added after every third rat to keep a constant halothane concentration. After loss of consciousness, the rats are placed in a Tempedair® drier before being returned to their cages. The animals are then left undisturbed until day 7.

4.4.3.1.4 Restress-session

One week after the initial stressor, the rats are exposed to a 're-stress' session, involving 20 minute forced swim in the same conditions as the second session of the initial stressor (25 cm ambient water (25°C) in a Porsolt swim tank). Thereafter, the rats are again dried in the Tempedair® drier and returned to their cages.

All TDS-stress procedures take place between 08h00-13h00.

4.5 Pharmacological studies

4.5.1 Drugs and drug administration

Five groups of rats (n=10/group) were utilized in the first pharmacological study. Treatments for this study included pyrollidine dithiocarbamate (PDTC), 7-nitroindazole monosodium salt (7-NINA), memantine hydrochloride, sildenafil and saline. All drugs were dissolved in saline (0.9% NaCl) and administered i.p at the dosage and protocols described in Table 4-1. Drugs and vehicle were administered at 08h00 each morning.

Table 4-1: Drug treatment, dosage and duration.

Group number	Name of drug	Treatment dose	Duration of treatment	Reference
1	PDTC	70mg/kg/d	7 days	Madrigal *et al.*, 2003
2	7-NINA	20mg/kg/d	7 days	Volke *et al.*, 1997
3	Memantine	5mg/kg/d	7 days	Rogoz *et al.*, 2002
4	Sildenafil	10mg/kg/d	7 days	Ferrari *et al.*, 2002
5	Saline		7 days	

All groups were treated daily with the appropriate drugs for a total of 7 days. The drug treatments started on day 7 ps (Figure 4-2) and continued for 7 days until day 14 when the animals were sacrificed by decapitation. The hippocampus of each rat brain was quickly removed and frozen in liquid nitrogen (-196°C). The tissues were subsequently stored at -86°C until used for later assay.

Additionally, five groups of rats (n=6/group) were utilized in the second pharmacological study where the same drug treatments were used. PDTC, 7-NINA, memantine and sildenafil were dissolved in saline (0.9% NaCl) and administered i.p each morning at 08h00, at the dosages and protocols described in Table 4-1. A control group received saline injection i.p. The groups were treated for 7 days without stress and sacrificed on day 7. The hippocampus of each rat brain was quickly removed and frozen in liquid nitrogen (-196°C). The tissues were subsequently stored at -86°C until used for later assay.

4.5.2 Tissue extraction

After the animals were sacrificed by decapitation, the appropriate brain regions were rapidly dissected on an ice-cooled dissection slab. The hippocampi were removed, immediately fixed in liquid nitrogen (-196°C) and stored at -86°C until the assays were performed.

4.5.3 Tissue preparation

On the day of the NOS, NO_x and cGMP assays, the hippocampi were removed from -86°C storage and allowed to thaw at room temperature.

4.5.3.1 NOS assay

The tissues were suspended in 1 ml homogenising buffer (pH = 7.2) containing 25mM Tris, 1mM EDTA and 1mM EGTA, homogenised with a Heidolph glass-teflon homogenizer (15 strokes on ice) and centrifuged at 10 000 rpm for 30 minutes at 2-4°C using a refrigerated, desk centrifuge 3K15. The supernatant was separated from the tissue pellet and kept on ice until used.

4.5.3.2 NO_x assay

The tissues were suspended in 1 ml homogenising buffer (pH = 7.2) containing 25mM Tris, 1mM EDTA and 1mM EGTA, homogenised with a Heidolph glass-teflon homogenizer (15 strokes on ice) and centrifuged at 14 000 rpm for 10 minutes at 2-4°C using a refrigerated, desk centrifuge 3K15. The supernatant was separated from the tissue pellet and kept on ice until used.

4.5.3.3 cGMP assay

The tissue samples were deproteinized by using the Simple buffer extraction method (Amersham, UK). Each sample is extracted by homogenization (Heidolph glass-teflon homogenizer) in a buffer containing 4mM EDTA (to prevent enzymatic degradation of cGMP), followed by heating at 100°C for 3 minutes to coagulate protein. Thereafter, the homogenate was cooled on ice and centrifuged at 2000g for 45minutes using a refrigerated desk centrifuge 3K15. The supernatant was separated from the tissue pellet, kept on ice and used for determination of cGMP.

The heat conditions and Ca^{2+}-chelation are necessary to prevent post-mortem changes in cyclic nucleotide levels by inhibiting the action of synthesizing (NOS, sGC) and hydrolytic (phosphodiesterase) enzymes through heat degradation and restriction of co-factor and pH requirements (Harvey *et al.*, 1994).

4.5.4 NOS assay

Determination of hippocampal NOS activity was determined as described by Bredt & Schmidt, 1996.

1M Tris (pH=7.4), 1mM BH_4, 4mM DTT, 0.1mM FAD, 0.1mM FMN, 10mM NADPH, 10 μg / $\mu \ell$ calmodulin, 5 μM L-[^{3}H]arginine, and 125mM $CaCl_2$ were incubated for 2 minutes at 37°C. Thereafter, tissue homogenates containing approximately 50 μg protein were added to the reaction cocktail and incubated for 15 minutes at 37°C. After 15 minutes, the samples were removed from incubation and the reaction stopped by dilution with 500 $\mu \ell$ of ice-cold stop-buffer (pH= 5.5), containing 50mM HEPES and 5mM EDTA. The product of the enzymatic reaction, L-[^{3}H]citrulline, was then separated by chromatography on columns filled with 1ml Dowex 50W×8 resin, which retains arginine. The columns were standardised with 3×1 ml stop-buffer. Thereafter, 2×0.5ml stop-buffer was added and the radioactive citrulline collected in scintillation vials and mixed with an appropriate scintillation cocktail for counting by a Packard United Technologies, Tri-Carb 2100 TR Liquid scintillation analyzer. Blanks contained all the reagents except the tissue homogenates and were used to determine the extent of L-[^{3}H]citrulline formation in the absence of NOS activity. Only L-[3H]arginine (5 $\mu \ell$) was used to determine total counts per picomole.

The assays were performed in the presence of $CaCl_2$ to determine both Ca^{2+}-dependent (eNOS + nNOS) and -independent NOS (iNOS) activity, thus representing total NOS activity. Total NOS activity was expressed as picomoles citrulline formed per mg protein present in the homogenate per minute (pmol/mg/min).

4.5.4.1 Protein determination

The protein concentration of the tissue homogenate carried out on the day of the NOS assay, was determined by the method described by Lowry *et al.* (1951), and outlined below:

A specific volume of the tissue homogenate was added to a solution of copper (Cu^{2+})-ions in an alkaline (NaOH) medium. This solution was then incubated at room temperature. During the incubation period the Cu^{2+}-ions interact with the proteins in the tissue homogenate. Thereafter, a certain amount of Folin-Ciocalteus reagent

was subsequently added to this mixture, and the mixture incubated in a water bath at 50°C. The copper-protein complex acts as an electron donor and reduces the added reagent from yellow to deeply coloured tungsten blue. The concentration of protein is directly proportionate to the intensity of the colour, as determined by absorption spectrometry.

A protein sequence was made form bovine serum albumin (BSA) (1mg/ml) in volumes of $10\,\mu\ell$, $20\,\mu\ell$, $30\,\mu\ell$, $40\,\mu\ell$ and $50\,\mu\ell$. This was used as standard concentrations for the determination of protein concentration in the homogenate sample. The volume of the standard protein concentration was made up to 1ml with deionised, ddH_2O.

For the construction of a standard curve, six test tubes were filled according to the following protocol:

Table 4-2: Standards for sample protein determination.

Name of Tube	**ddH_2O ($\mu\ell$)**	**Homogenising buffer ($\mu\ell$)**	***BSA ($\mu\ell$)**
Blank	950	50	———
Standard 1	940	50	10
Standard 2	930	50	20
Standard 3	920	50	30
Standard 4	910	50	40
Standard 5	900	50	50
		Supernatant containing protein ($\mu\ell$)	
Unknown	950	50	———

* $10\,\mu\ell$ BSA = $10\,\mu g$ of protein, etc.

For the determination of protein concentration of the tissue homogenates, $50\,\mu\ell$ of the tissue homogenate was added to $950\,\mu\ell$ deionised, ddH_2O. To each tube, 1ml freshly prepared tartrate solution (10% Na_2CO_3 in 0.5M NaOH) was subsequently added to both the standard sequence and the tissue homogenate. This solution contained 1% potassium tartrate, 5% copper sulphate and 10% sodium bicarbonate

in 0.5M sodium hydroxide. The mixture was then incubated at room temperature for 10 minutes in order for the Cu^{2+}-ions to form a complex with the proteins. Thereafter, 3ml of Folin-Ciocalteus reagent (diluted 1:10 with ddH_2O) was added to the mixture, followed by 10 minute incubation at 50°C in a water bath. The mixture was then allowed to cool to room temperature and absorbance (A_{650}) read at a wavelength of 650nm using a Spectronic 20 (Bausch and Lomb) spectrophotometer. Homogenate protein concentrations were then extrapolated from a standard curve of A_{650} against protein (BSA) concentration.

4.5.5 cGMP assay

Cyclic GMP levels were determined using a radio-immunoassay method described by Amersham Ltd, Buckinghamshire, U.K.

14 assay tubes (suitable for centrifuging) and additional tubes for unknowns, in duplicate, were set up and kept in ice throughout the assay. The tubes were labelled and arranged according to the protocol shown in Table 4-3. 50 $\mu\ell$ titrated [8-^{3}H] cyclic GMP was pipetted into all the tubes. 100 $\mu\ell$ of the Tris-EDTA buffer was added to tubes 1 and 2, whereas tubes 13 and 14 each contained 100 $\mu\ell$ of blank reagent. Starting with the lowest level of standard cGMP, 100 $\mu\ell$ of each cGMP serial dilution (0.0625 - 1pmol) was pipetted into five pairs of tubes respectively, to provide a standard calibration curve. The remainding tubes (15, etc.) received 100 $\mu\ell$ (in duplicate) of each separate unknown. After 50 $\mu\ell$ of antiserum was added to all the tubes, it was vortex mixed, placed back onto ice and refrigerated at 2-8°C for 1.5 hours. After incubation, a 60% saturated, ice-cold ammonium sulphate solution was added to all the tubes, mixed and allowed to stand for 5 minutes (on ice). After the final incubation, the antibody bound fraction was separated by centrifugation at 12 000rpm for 2 minutes at 4°C. The resultant supernatant liquid was decanted and discarded from each tube followed by adding 1.1 ml distilled water to dissolve the sediment. After the tubes were mixed and the precipitate dissolved, 1ml aliquots were removed from each tube, placed into a counting vial (containing a water soluble scintillant) and the radioactivity measured using scintillation counting (Packard United Technologies, Tri-Carb 2100 TR Liquid scintillation analyzer).

The competition between unlabelled cGMP and a fixed quantity of the tritium labelled compound for binding to an antiserum form the basis of this assay. The antiserum has a high affinity and specificity for cGMP. The amount of labelled cGMP bound to the antiserum is inversely related to the amount of cGMP present in the sample. Ammonium sulphate / $(NH_4)_2SO_4$ is used in the assay to separate the antibody-bound cGMP from the unbound nucleotide, followed by centrifugation. The precipitate containing the antibody-bound complex is dissolved in water and its activity determined by liquid beta-scintillation counting. The concentration of unlabelled cGMP in the sample is then determined from a linear standard curve.

Table 4-3: Cyclic GMP assay protocol.

Tube nr	**Tris-EDTA buffer** ($\mu\ell$)	**Assay blank**	**[^{3}H] cGMP** ($\mu\ell$)	**Antiserum** ($\mu\ell$)	**Standard** ($\mu\ell$)
*1,2	100	—	50	50	Zero
**3,4	—	—	50	50	**E** (100 $\mu\ell$)
5,6	—	—	50	50	**D** (100 $\mu\ell$)
7,8	—	—	50	50	**C** (100 $\mu\ell$)
9,10	—	—	50	50	**B** (100 $\mu\ell$)
11,12	—	—	50	50	**A** (100 $\mu\ell$)
13,14	—	100	50	—	**Blank** ($\mu\ell$)
					Unknowns ($\mu\ell$)
15,16	—	—	50	50	100
17,etc	—	—	50	50	100

Cyclic GMP standards / original solutions:

Zero = 0 pmol **C** = 0.25 pmol

E = 0.0625 pmol **B** = 0.5 pmol

D = 0.125 pmol **A** = 1.0 pmol

*Tubes 1 and 2, containing Tris-EDTA buffer, are for the determination of zero-dose binding or binding in the absence of unlabelled cGMP.

**Tubes 13 and 14, containing the blank reagent, are used for determining the assay blank (blank counts per minute).

4.5.5.1 Protein determination

The Bradford protein assay was used as described by Bradford (1976) to determine protein in tissue homogenates between 0.1-2mg/ml using Bovine serum albumin (BSA) as standard (2mg/1ml).

Seven test tubes, with a final volume of 100 $\mu\ell$, were each filled with homogenising buffer (HB) and BSA. For the construction of the standard curve, the protocol in Table 4-4 was used.

Table 4-4: Protein standards.

Protein concentration (mg/ml)	Dilution in test tubes	
	Volume BSA (2mg/ml) ($\mu\ell$)	Volume of HB ($\mu\ell$)
0	-------	100
0.1	5	95
0.4	20	80
0.7	35	65
1.0	50	50
1.4	70	30
2.0	100	-------

Preparation of the 96-well plate

A sufficient quantity of the Bradford reagent (Sigma-Aldrich) was withdrawn and brought to room temperature. 5 $\mu\ell$ (in duplicate) of each tube (blank, standards and unknowns) were added to separate wells of a 96-well plate. Also in duplicate, 250 $\mu\ell$ of the Bradford reagent were added to each well, using a micropipette. Immediately thereafter, the plate was mixed for $\pm$ 30 seconds, using the shaking facility of the plate-reader and left to incubate (at room temperature) for $\pm$ 15 minutes. The absorbance (A_{560}) was read at a wavelength of 560nm by means of a Labsystem Multiskan RC spectrophotometer plate-reader. The net absorbance values were determined by subtracting the average blank absorbance value from all the other absorbance values. Homogenate protein concentrations were then determined by plotting the net absorbance against protein concentration of the standards.

4.5.6 NO_x assay

The assay of serum NO_3^- and NO_2^- (collectively referred to as nitrogen oxides or NO_x) was performed using a Griess procedure previously described by Verdon *et al.* (1995) as modified by Titheradge, 1998.

4.5.6.1 The Griess reaction

The concentration of NO_3^- plus NO_2^- in samples is measured by a two-step procedure. The NO_3^- is first enzymatically reduced to NO_2^- using a NADPH-dependent nitrate reductase from *Aspergillus* species, followed by measurement of the total NO_2^- in the sample by a stoichiometric diazotization reaction using the Griess reagent to form a purple-azo product. The original NO_2^- concentration in the samples can be determined in samples in which the NO_3^- has not been converted, thus allowing the measurement of NO_3^- by difference.

Eleven test tubes, with a final volume of 1000 $\mu\ell$, were each filled with homogenising buffer (HB) and 10mM nitrate standard stock solution (85mg sodium nitrate/100ml ddH_2O). For the construction of the standard curve, the protocol in Table 4-5 was used.

Table 4-5: Preparation of standards.

[Std] (μM)	Volume stock solution ($\mu\ell$)	Volume HB ($\mu\ell$)	Total volume ($\mu\ell$)
100	10	990	1000
90	9	991	1000
80	8	992	1000
70	7	993	1000
60	6	994	1000
50	5	995	1000
40	4	996	1000
30	3	997	1000
20	2	998	1000
10	1	999	1000
0	0	100	1000

Total NO_2^- plus NO_3^- were determined using the NADPH-dependent nitrate reductase assay and 96-well plates (Table 4-6). Briefly, 50 $\mu\ell$ of each sample and

nitrate standard is pipetted into the 96-plate in duplicate. Thereafter, 40 $\mu\ell$ of the conversion buffer, consisting of 3.8mg glucose-6-phosphate in 5 $m\ell$ 14mM sodium phosphate buffer, 2 units glucose-6-phosphate dehydrogenase and 1 unit NADPH-dependent nitrate reductase, was added into each well. The sodium phosphate buffer consisted of 101mg $Na_2HPO_4.2H_2O$ + 20.8mg $NaH_2PO_4.2H_2O$ in 50 $m\ell$ ddH_2O. This was followed by adding 10 $\mu\ell$ 10 μM reduced NADPH solution, consisting of 1.7mg NADPH in 2 $m\ell$ ddH_2O (diluted 100-fold), into each well. The well plate was mixed, using the shaking facility of the plate-reader and left for incubation (at room temperature) for $\pm$ 45 minutes, to convert NO_3^- to NO_2^-. After incubation, 100 $\mu\ell$ of Griess working solution were added to each well. This solution was prepared by mixing one part Griess reagent A (0.1g NEDA in 100 $m\ell$ ddH_2O) with one part Griess reagent B (1g sulphanilamide in 100 $m\ell$ 5% v/v orthophosphoric acid) immediately before using. The well was again mixed and left for 15 minutes to incubate at room temperature to allow colour development. Thereafter, the absorbance (A_{560}) was read at a wavelength of 560nm by means of a Labsystem Multiskan RC spectrophotometer plate-reader and the original amount of NO_2^- was calculated by comparing it with the NO_2^- standard curve.

4.5.6.2 Protein determination

Total cell protein was determined by using the Bradford protein assay (refer to section 4.6.5.1) as described by Bradford (1976) to normalize data as pmol nitrite/ μg of protein.

4.5.7 Statistical analysis

All data were analysed using a one-way analysis of variance (ANOVA). If significant differences were noted, post hoc analysis was performed using either Tukey's test (pairwise comparison of the means of different groups) or Dunnett's test (comparison between the control group and experimental group) and linear regression. Prism version 4 software was used for graphic presentation and statistical analysis. Data is presented as means $\pm$ S.E.M. (duplicate or triplicate).

In the Control versus TDS study, the NOS data was analyzed using the Student's T-test (Graphpad, Prism 4).

In the first pharmacological study (Control versus TDS versus drug treatment group), both cGMP- and NO_x levels were determined by using the Tukey's test (Kramer, 1956), whereas in the second pharmacological study (Control versus different drug treatment groups), for both cGMP- and NO_x levels, the Dunnett's test was applied according to Miller (1980).

In all cases, a confidence interval of 95% ($p<0.05$) was applied.

Chapter 5 Results

5.1 Time-dependent sensitisation (TDS)

5.1.1 Hippocampal NOS-activity and NO_x accumulation

The initial characterisation of the NOS assay with regards to co-factors, optimum protein concentration and assay conditions was performed by Oosthuizen (2003). To establish the optimum substrate (L-arginine) concentration for the current study, a concentration series using 50 µg protein was set up. A representative standard curve is depicted in Figure 5-1.

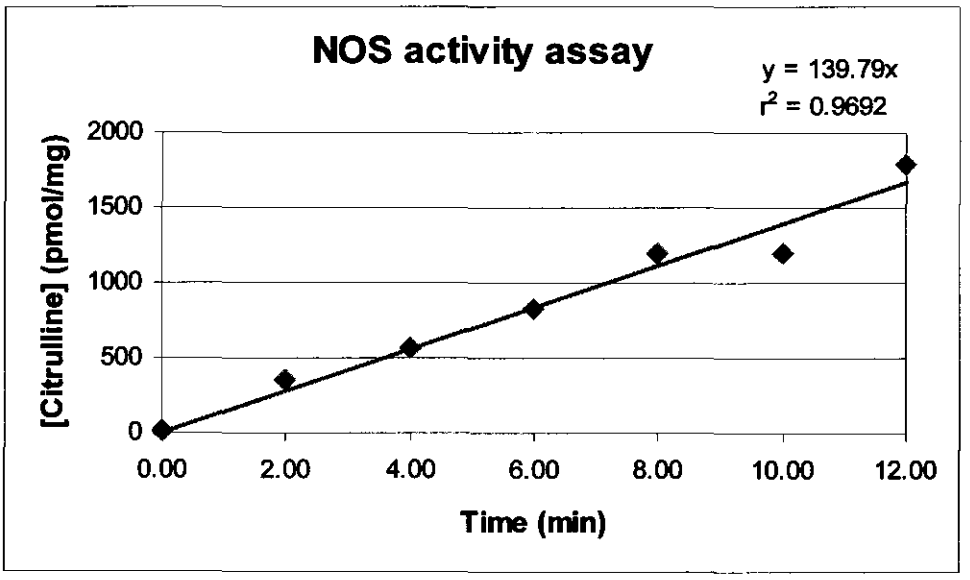
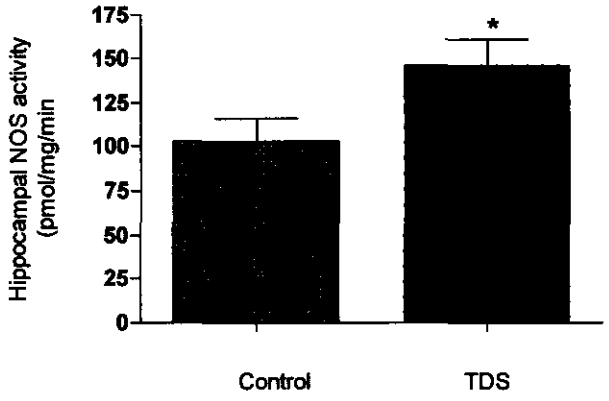


Figure 5-1: A representative standard curve of the NOS assay.

Using the treatment and assay methods described in section 4.5.4, total hippocampus NOS activity was found to be both reliable and accurate. In order to confirm earlier data obtained in this laboratory (Oosthuizen, 2003; Harvey *et al.*, 2004a), the two groups, namely TDS (n=10) versus control (n=10) were compared. Formal two-sample t-tests, determining the differences in the mean of both the

control group and the TDS groups, revealed a significant difference across the two groups. ($p<0.05$; Figure 5-2).

Clearly, TDS evoked a significant increase in total hippocampal NOS activity, concordant with earlier studies (Oosthuizen, 2003; Harvey *et al.*, 2004a).



	mean ± SEM pmol / mg / min
Control	103.3420 ± 40.03158
TDS	146.2700 ± 44.58630*

*p = 0.036 vs control (Student's t-test)

Figure 5-2: The effect of TDS-stress on total hippocampal NOS activity. Descriptive statistics are provided in the adjacent table.

In order to confirm and extend these findings, two similarly treated groups (n=10/group) were set up, except now hippocampal brain extracts were analyzed for accumulation of the stable oxidative metabolites of NO, namely NO_2^- and, NO_3^-, collectively referred to as nitrogen oxides (NO_x). Initial characterisation of the NO_x assay, as described in section 4.5.6, was performed by Nel (2003). A representative standard curve for hippocampal NO_x is depicted in Figure 5-3. Formal two-sample t-tests performed on the data found that TDS-stress induced a significant increase in hippocampal NO_x accumulation composed to control ($p=0.000195$; Figure 5-4).

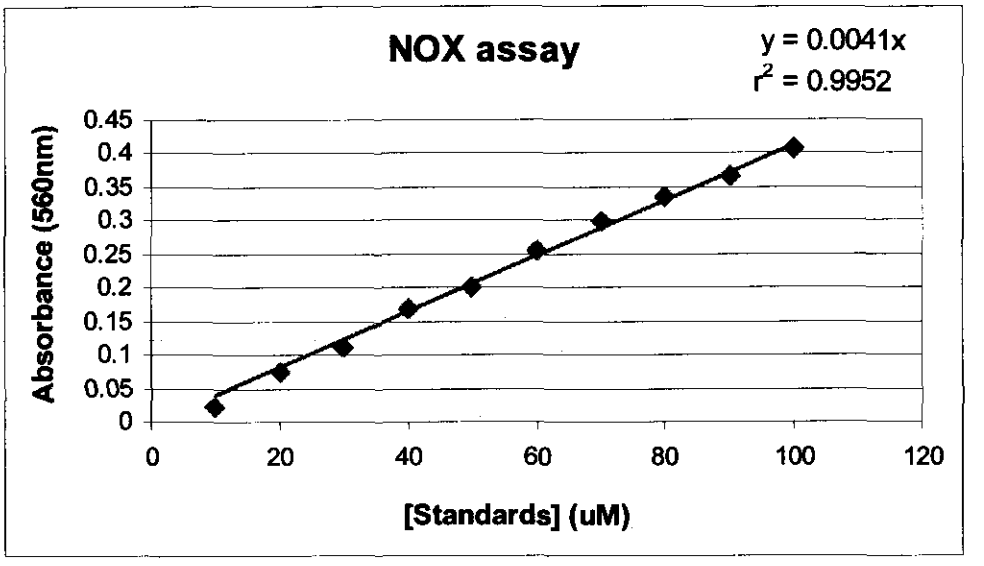
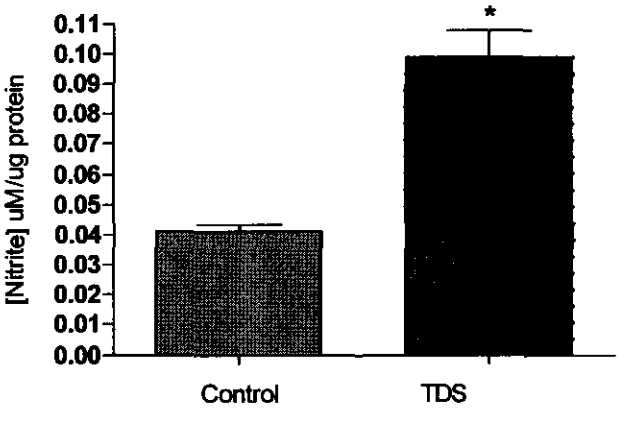


Figure 5-3: Representative standard curve of the nitrite/nitrate (NO_x) assay.



	mean ± SEM uM / ug / protein
Control	0.04087 ± 0.002479
TDS	0.09881 ± 0.008898*

*p = 0.000195 vs control (Student's t-test)

Figure 5-4: The effect of TDS-stress on total hippocampal NO_x accumulation. Descriptive statistics are provided in the adjacent table.

5.2 Pharmacological studies

Clearly, TDS-stress activates the NO-pathway, evident in both an increase in total NOS activity as well as an accumulation of NO_x on day 7 ps. In the following series of studies, changes in NO-pathway activity post TDS-stress was modulated with agents that act selectively on various targets within the NO-pathway. For the purpose of the current investigation, the selective nNOS inhibitor, 7-NINA, the selective PDE-5 inhibitor, sildenafil, the NMDA receptor antagonist, memantine, and the inhibitor of the iNOS transcription factor NF$\kappa\beta$, PDTC, was used. These drugs effectively target the NO-pathway at the level of enzyme-substrate dependency (7-NINA), activation of GC-cGMP downstream (sildenafil), activation of NOS more upstream at the level of gene activation (PDTC), and finally activation of the NO-pathway via glutamate receptor activation (memantine).

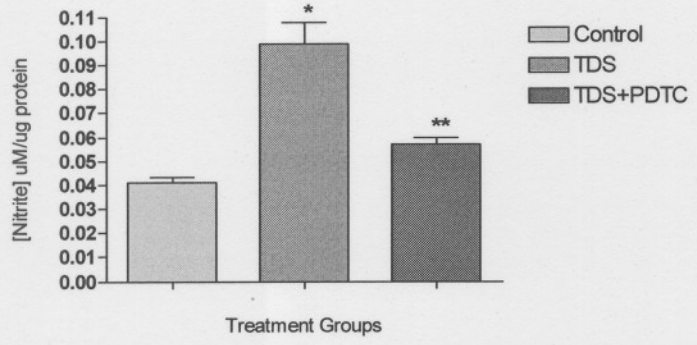
Rats, weighing 200-250g, were separately treated with the 4 different modulators of the NO-cGMP pathway for seven days as described in section 4.5.1. For the purpose of the pharmacological studies and since NOS and NO_x data after TDS-stress was congruent with activation of NOS, only tissue NO_x was analyzed. However, to consolidate these findings, and to concur with the use of cGMP-dependent drugs used in this section, the analysis of hippocampal cGMP levels was added to these studies.

5.2.1 Effects of TDS with / without drug treatment

5.2.1.1 NO_x assay data

5.2.1.1.1 Role of NF$\kappa\beta$ in the NO_x response to TDS

One way analysis of variance of the data revealed significant differences across the groups [$F(6,108)=19.39697$, $p<0.000001$]. TDS-stress engendered a significant increase in NO_x on day 7 ps (Figure 5-5; Tukey's test; $p=0.000195$), that was completely blocked by the administration of PDTC during stress (Figure 5-5; Tukey's test; $p=0.000283$).



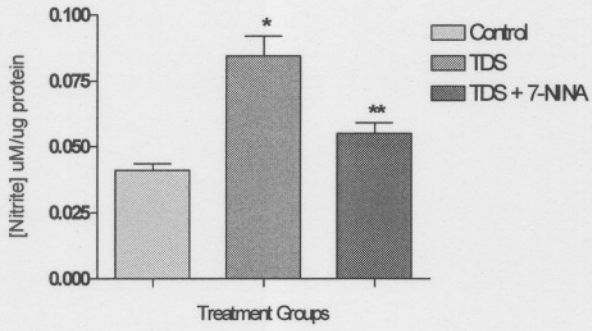
	mean ± SEM uM/ug protein
Control	0.040870 ± 0.002479
TDS	0.098810 ± 0.008898*
TDS+ PDTC	0.057060 ± 0.002853**

*p = 0.000195 vs control **p = 0.000283 vs TDS

Figure 5-5: The effect of TDS alone and with a sub-chronic challenge with PDTC during stress on hippocampal NO_x levels. Descriptive statistics are provided in the adjacent table.

5.2.1.1.2 Role of nNOS in the NOx response to TDS-stress

As described earlier, TDS-stress engendered a significant increase in NO_x on day 7 ps (Figure 5-6: Tukey's test; p=0.000328), that was completely blocked by concomitant administration of 7-NINA during the stress period (Figure 5-6; Turkey's test; p=0.000169).



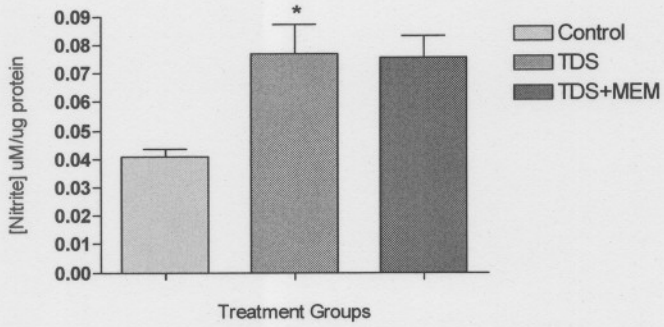
	mean ± SEM uM/ug protein
Control	0.040870 ± 0.002479
TDS	0.083970 ± 0.007472*
TDS + 7-NINA	0.055300 ± 0.00383**

*p = 0.000328 vs control **p = 0.000169 vs TDS

Figure 5-6: The effect of TDS alone and with a sub-chronic challenge with 7-NINA during stress on hippocampal NOx levels. Descriptive statistics are provided in the adjacent table.

5.2.1.1.3 Role of NMDA receptor activation in the NOx response to TDS-stress

TDS-stress again engendered a significant increase in NO_x on day 7 ps (Figure 5-7; Tukey's test; p=0.005107). Memantine, however, failed to modilfy the NO_x response to TDS-stress (Figure 5-7).



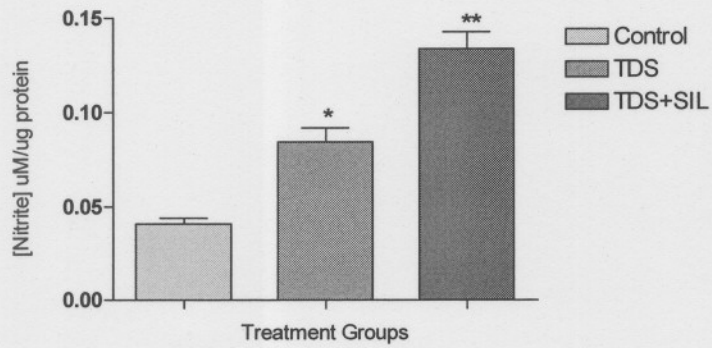
	mean ± SEM uM/ug protein
Control	0.040870 ± 0.002479
TDS	0.077000 ± 0.010260*
TDS + MEM	0.075540 ± 0.007522

*p = 0.005107 vs control

Figure 5-7: The effect of TDS-stress alone and with a sub-chronic challenge with memantine during stress on hippocampal NO_x levels. Descriptive statistics are provided in the adjacent table.

5.2.1.1.4 Role of cGMP in the NOx response to TDS-stress.

TDS-stress evolved a significant increase in NO_x on day 7 ps (Figure 5-8; Tukey's test; p=0.000127), while concomitant administration of the PDE-5 inhibitor, sildenafil, significantly increased NO_x vs TDS alone (Figure 5-8; Tukey's test; p=0.001075).



	mean ± SEM uM/ug protein
Control	0.040870 ± 0.002479
TDS	0.084500 ± 0.007478*
TDS+Sil	0.134000 ± 0.008261**

*p = 0.000127 vs control **p = 0.001075 vs TDS

Figure 5-8: The effect of TDS-stress alone and with a sub-chronic challenge with sildenafil during stress on hippocampal NOx levels. Descriptive statistics are provided in the adjacent table.

5.2.1.2 cGMP assay data

One way analysis of variance of the data revealed significant differences across the groups [F(6,108)=18,52001, p < 0.000001]. A representative standard curve for the cGMP assay is provided in Figure 5-9, indicating a linear regression over the cGMP concentration range based on preliminary pilot studies to determine an ideal concentration range for this study.

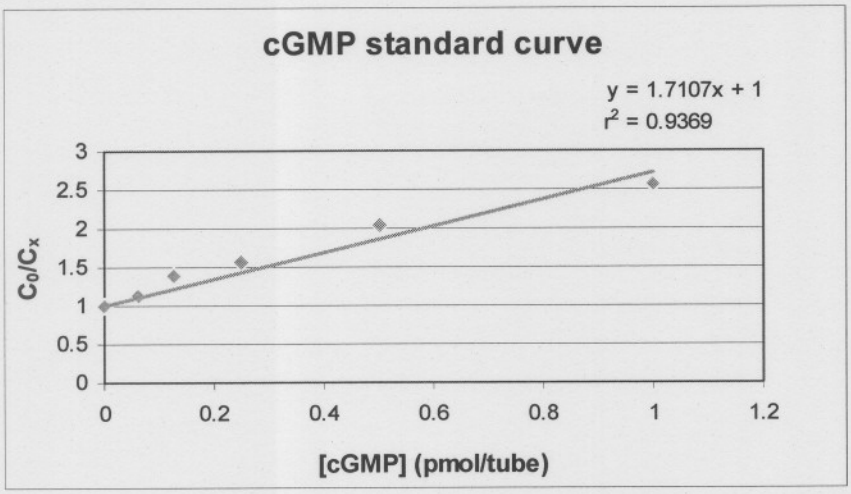
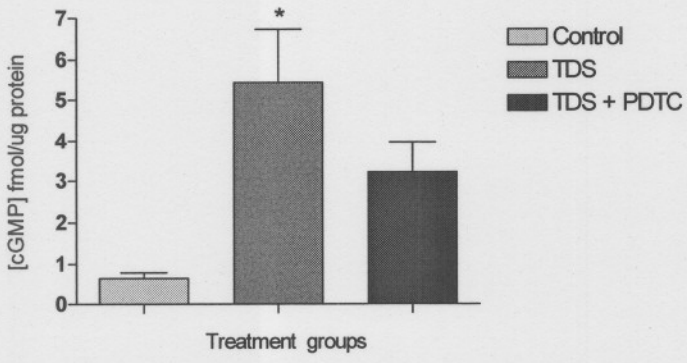


Figure 5-9: A representative standard curve for the cGMP assay.

5.2.1.2.1 Role of NFκβ in the cGMP response to TDS.

TDS-stress evoked a significant increase in hippocampal cGMP on day 7 ps (Figure 5-10; Tukey's test; p=0.000376). PDTC treatment during stress inhibited this response, although this narrowly missed significance (Figure 5-10).



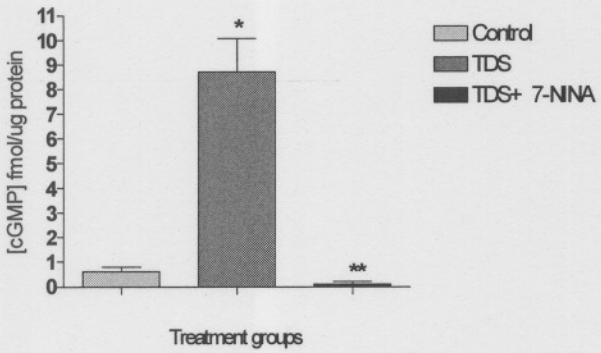
	mean ± SEM fmol/ug protein
Control	0.630990 ± 0.149929
TDS	5.646104 ± 1.153123*
TDS+ PDTC	3.351636 ± 0.663436

*p = 0.000376 vs control

Figure 5-10: The effect of TDS-stress alone and with a sub-chronic challenge with PDTC during stress on hippocampal cGMP levels. Descriptive statistics are provided in the adjacent table.

5.2.1.2.2 Role of nNOS in the cGMP response to TDS-stress.

TDS-stress evoked a significant increase in hippocampal cGMP on day 7 ps (Figure 5-11; Turkey's test; p=0.000127). Administration of 7-NINA during stress was completely effective in blocking this response (Figure 5-11; Tukey's test; p=0.000127).



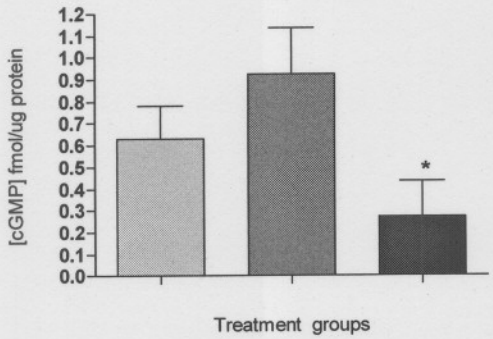
	mean ± SEM fmol/ug protein
Control	0.630990 ± 0.149929
TDS	8.688830 ± 1.375220*
TDS+ 7-NINA	0.100997 ± 0.099028**

*p = 0.000127 vs control **p = 0.000127 vs TDS

Figure 5-11: The effect of TDS-stress alone and with a sub-chronic challenge with 7-NINA during stress on hippocampal cGMP levels. Descriptive statistics are provided in the adjacent table.

5.2.1.2.3 Role of glutamate-NMDA receptor in the cGMP response to TDS- stress.

TDS-stress again induced an increase in cGMP in the hippocampus in rats on day 7 ps, although in this group significance was not attained (Figure 5-12; Tukey's test). Memantine, however, significantly decreased hippocampal cGMP compared to that evoked in the TDS group alone (Figure 5-12; Tukey's test; p=0.035487).



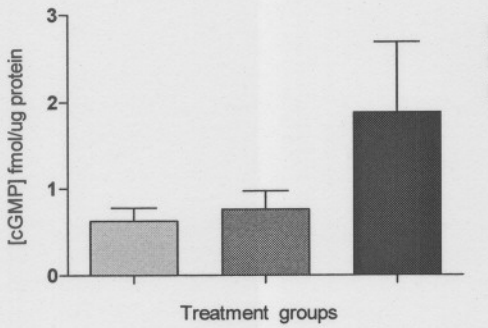
	mean ± SEM fmol/ug protein
Control	0.63099 ± 0.149929
TDS	0.92536 ± 0.206911
TDS+MEM	0.268011 ± 0.167006 *

*p = 0.035487 vs memantine

Figure 5-12: The effect of TDS-stress alone and with a sub-chronic challenge with memantine during stress on hippocampal cGMP levels. Descriptive statistics are provided in the adjacent table.

5.2.1.2.4 Role of cGMP in the cGMP response to TDS.

In this group, TDS-stress failed to evoke a profound effect on hippocampal cGMP as in previous groups (Figure 5-13; Tukey's test). Sildenafil during stress increased hippocampal cGMP accumulation, although the increase failed to reach significance (Figure 5-13; Tukey's test).



	mean ± SEM fmol/ug protein
Control	0.63099 ± 0.149929
TDS	0.71538 ± 0.191068
TDS + Sil	1.87783 ± 0.809243

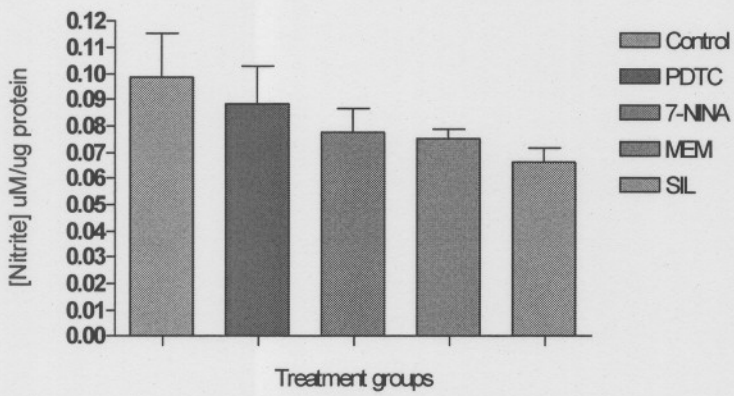
Figure 5-13: The effect of TDS-stress alone and with a sub-chronic challenge with sildenafil on hippocampal cGMP levels. Descriptive statistics are provided in the adjacent table.

5.2.2 Control versus different drug treatment groups

This analysis was performed to determine whether the various drug treatments were able to modify the NO-cGMP response under control conditions, i.e. in the absence of stress. All rats were treated for 7 days.

5.2.2.1 NO_x assay data

One way analysis of variance of the data failed to reveal differences across the groups [F (4;22)= 1.099951; p<0.381354]. Further analysis using the Dunnett's test demonstrates that neither drug alone significantly modified hippocampal NO_x, although both sildenafil and memantine showed a distinct tend to decrease NO_x.



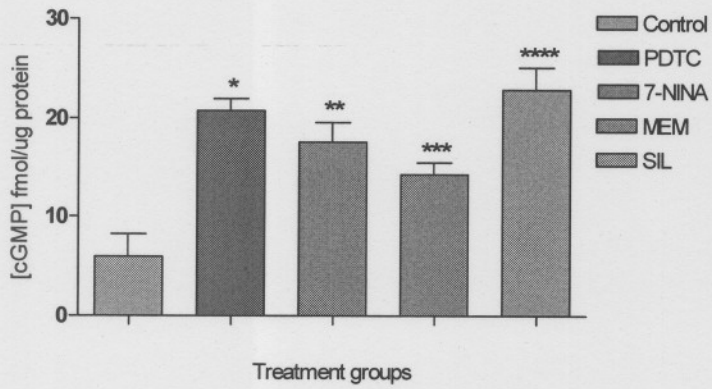
	mean ± SEM uM/ug protien
Control	0.098467 ± 0.016756
PDTC	0.088209 ± 0.014521
7-NINA	0.077673 ± 0.009051
MEM	0.075306 ± 0.003415
Sil	0.066069 ± 0.005502

Figure 5-14: Hippocampal NO_x values for the different drug treatment regimens compared to control. Descriptive statistics are provided in the adjacent table.

From these data it can be confined that none of the drugs were able to modify the basal No_x response in healthy, unstressed animals.

5.2.2.2 cGMP assay data

One way analysis of variance of the data revealed significant differences across the groups [F(4;24)=13.49576, p<0.000007]. Further analysis using the Dunnett's test demonstrated that all the drugs alone significantly increased hippocampal cGMP.



	mean ± SEM fmol/ug protein
Control	5.966978 ± 2.270238
PDTC	20.68007 ± 1.268937*
7-NINA	17.55762 ± 1.927109**
MEM	14.304400 ± 1.204096***
Sil	22.88704 ± 2.199383****

*p = 0.000014 vs control
**p = 0.000812 vs control
***p = 0.011196 vs control
****p = 0.000000 vs control

Figure 5-15: Hippocampal cGMP values for the different treatment regimens in the second sub-chronic study compared to control. Descriptive statistics are provided in the adjacent table.

From these data it is evident that all four drugs on their own evoked a significant increase in hippocampal cGMP in normal unstressed animals.

Chapter 6 Discussion

Results obtained from this study indicate that TDS-stress significantly increased hippocampal NOS activity, NO_x levels and cGMP levels. In the pharmacological study, the observed increase in NO_x was blocked by treatment with either PDTC or 7-NINA, while memantine was without effect. Sildenafil significantly augmented stress-induced NO_x production. Focusing on hippocampal cGMP accumulation, 7-NINA and memantine significantly blocked the increase in cGMP evoked by TDS-stress, with PDTC attenuating this response, but not significantly. Sildenafil, as with its effects on NO_x, increased hippocampal cGMP but not significantly. Additionally, administration of each drug separately for seven days without exposure to TDS-stress, did not evoke significant changes in NO_x levels, compared to the control group. However, significant increases in cGMP levels, compared to the control groups, was found with all four drugs.

PTSD is a severe disorder that develops and worsens over time (NCPTSD, 2000). Several neurotransmitters and second messengers are involved in the abnormal behavioural responses characteristic of PTSD (van der Kolk, 1994). Because of the putative important role of NO in the stress response, the primary aim of this project was to investigate the role of the NO-cGMP pathway in the neurobiology of PTSD using an animal model that emphasizes repeated trauma , and to evaluate the effects of stress on NO and cGMP levels and its response to drugs that modulate the NO-cGMP pathway.

The NO-sGC-cGMP pathway has been implicated in anxiety (Faria *et al.*, 1997; Li & Quock, 2002; Volke *et al.*, 2003) and anxiety-related disorders such as PTSD (Pall, 2003). Moreover, PTSD is thought to be induced by excessive NMDA receptor stimulation, a pathway that is known to produce excessive NOS activation and release of NO, as well as the pro-oxidant, peroxynitrite ($ONOO^-$) (Pall, 2001). Yeh and colleagues (2002) investigated the correlation of NO with acute stress disorder (ASD) in human subjects and found a significant inverse correlation between severity of stress symptoms and the concentration of NO in the serum. Indeed, PTSD may involve mechanisms that center around the release of excessive NO and $ONOO^-$ (Oosthuizen *et al.*, 2005). PTSD presents with evidence of neurodegenerative

pathology, including shrinkage of the hippocampus that is correlated with cognitive deficits (Lyons, 2002). The neurotoxic action of NO (Leong *et al.*, 2002), as well as its central role in learning and memory (Iga *et al.*, 1993), may provide an explanation for the neuropathological characteristics of PTSD.

Using an animal model that emphasizes repeated trauma, Harvey *et al.* (2003) found that TDS-stress induces behavioural, neurochemical and cognitive anomalies in rats characteristic of PTSD. More recently, these authors also implicated a possible role for NO in this animal model and therefore, by implication, PTSD (Harvey *et al.*, 2004a). Both the above studies on TDS-stress focused on the hippocampus, a brain structure that is well known to modulate, and also be influenced, by severe traumatic stress. The hippocampus functions as a central point where neuro-chemical and neurohormonal responses to stress are integrated (Bremner, 1999), while it is mainly the hippocampus that suffers damage in PTSD. It is therefore the hippocampus that is the focus of this investigation.

In the present study, NO_x, the stable oxidative metabolites of NO, as well as intracellular cGMP levels, were monitored as an index of the functioning and modulation of the NOS/sGC pathway. In order to replicate earlier findings implementing NO in PTSD (Harvey *et al.*, 2004a), the first approach was to measure hippocampal NOS activity together with the hippocampal accumulation of NO_x in order to confirm a positive correlation between these parameters, and their possible relationship to TDS-stress in rodents.

Stress is known to be a key factor in the genesis of a number of neuropsychiatric disorders, while adaptation to stress helps in the coping process (Esch *et al.*, 2002). Pall (2003) concurs that NO may have a critical in role in PTSD. In this study statistically significant increases in NOS activity as well as significantly raised levels of the NO_x, and in some cases raised cGMP levels, were observed in experimental animals exposed to the TDS-model. Consistent with this, Harvey *et al.* (2004a) found an increase in the speed of the reaction (V_{max}) of NOS in the hippocampus of animals immediately after being exposed to the acute stressors of the TDS model, but which was sustained for up to three weeks after the TDS procedure. Masood *et al.* (2004) showed that repeated stress resulted in higher levels of brain nitrates and nitrites, collectively known as NO_x, as compared to that of a single restraint stress group. These results have far reaching implications, indicating that severe stress increases NOS, while a protracted elevation in NO-production in the hippocampus

after exposure to trauma may be causally related to hippocampal degeneration and subsequent memory deficits observed in PTSD and also after TDS-stress (Harvey *et al.*, 2003). This suggests that TDS-stress may be targeting one or more important modulatory cites involved in the NO-cGMP pathway, either at the receptor level, or more down-stream at the level of NOS itself or its activation by nuclear transcription factors.

Summarizing these data, TDS-stress resulted in a statistically significant increase in hippocampal NOS activity when compared to basal levels (Figure 5-2), while also engendering a statistically significant increase in hippocampal NO_x accumulation, compared to basal levels (Figures 5-4, 5-5, 5-6, 5-7, 5-8). These results were highly reproducible. Furthermore, hippocampal cGMP levels were also significantly elevated in the rats after exposure to TDS-stress, compared to basal levels (Figures 5-10 and 5-11), although this increase was not significant in a consistent manner, eg. Figures 5-12 and 5-13. While this lack of repeatability is disappointing, it may have its origin in one of the important qualities of the TDS model, viz. bi-directional expression of symptoms (Uys *et al.*, 2003). In their respective reviews, Yehuda and Antelman (1993) and later Uys and colleagues (2003) described the important qualities of the TDS model, including: (i) it can be induced by acute and chronic physical or psychological stressful events; (ii) it is dose-dependent; (iii) the consequences persist for long periods and increase with time; (iv) the effects can be either excitatory or inhibitory; (v) TDS shows bi-directional expression of symptoms, with marked interindividual variability. The development of PTSD after trauma is unpredictable, while symptoms may express themselves in opposite ways in different individuals dependent on prior stress history and post stressor adaptations, for example, enhanced or reduced responsiveness to environmental stimuli (Yehuda & Antelman, 1993) and hypocortisolemia (Sautter *et al.*, 2003) or hypercortisolemia (Baker *et al.*, 1999).

The afore mentioned results, nevertheless, indicate that increased levels of NO and cGMP may play an important role in the aetiology and pathophysiology of TDS-stress, and possibly PTSD. The second study was designed to evaluate the effect of select pharmacological manipulation of TDS-induced changes to hippocampal NO_x and cGMP levels. To this end, drugs selective for various targets within the NO-cGMP pathway were administered immediately after the sensitisation phase of the TDS procedure, including the NF$\kappa\beta$ antagonist, PDTC, the selective nNOS inhibitor,

7-NINA, the NMDA-receptor antagonist, memantine and the selective PDE-5 inhibitor, sildenafil.

One of the important activators of NOS, especially iNOS, is the nuclear transcription factor, NF$\kappa\beta$ (Lee *et al.*, 2004). PDTC, an inhibitor of NF$\kappa\beta$ (Lee *et al.*, 2004), significantly decreased NO_x as well as decreased cGMP levels in stressed animals (Figure 5-5) compared to that evoked by TDS-stress, although the latter missed significance (Figure 5-10). Since NF$\kappa\beta$ is primarily involved in the expression of iNOS, while cGMP originates from nNOS-mediated NO synthesis, this suggest that iNOS is the dominant isoform involved in TDS-induced NO-cGMP activation. This is supportive of that observed in an earlier study (Harvey *et al.*, 2004a).

PDTC is a potent immunomodulatory substance that modulates the inflammatory response *in vitro* and reduces mortality in endotoxic shock (Meisner *et al.*, 2000). The pathophysiological mechanism of the protective effect of PDTC *in vivo*, however, appears to be dual, comprising both antioxidative properties as well as the inhibition of NF$\kappa\beta$ (Schmidt *et al.*, 1995). Numerous binding sequences of NF$\kappa\beta$ on various genes with important immunologic functions characterize this transcription factor as a multi-potent regulatory factor in the inflammatory response (Liu *et al.*, 1997). PDTC is a water soluble, low-molecular weight substance that has been demonstrated to almost completely suppress NO production by immune-stimulated macrophages through inhibition of iNOS expression (Mülsh *et al.*, 1993) and to modulate lipopolisaccaride (LPS)-induced tumor necrosis factor alpha (TNF-α) production (Ziegler-Heitbrock *et al.*, 1993). This makes the startling suggestion that certain forms of stress, in particular PTSD, may represent an inflammatory condition of the brain. Certainly, the evidence for iNOS involvement strongly implicates inflammatory processes in TDS-stress and indeed, in PTSD (Oosthuizen *et al.*, 2005). Furthermore, two particular cytokines, IL-1β and IL-6, have been reported to be elevated in PTSD patients, providing some experimental support for the inflammatory process in the aetiology of PTSD (Maes *et al.*, 1999).

An important regulator of iNOS is NO itself. Studies have observed a reciprocal relationship between iNOS and nNOS, with NO produced by nNOS responsible for decreasing iNOS activity (Colasanti & Suzuki, 2000). Raised cGMP levels evoked by PDTC alone is intriguing, and possibly suggests that this increased cGMP, which invariably originates from nNOS-NO activation (Moreland *et al*,. 1998), may underlie

its effect to decrease TDS-induced NO_x accumulation. In other words, PDTC increases nNOS activity, that enhances the production of NO that, via two different pathways, leads to an increase in iNOS expression and cGMP levels, respectively. This is highly speculative and requires further investigation. However, it suggests that PDTC may under certain conditions, increase nNOS-mediated NO, which is neurotoxic. Indeed, neurotoxic and extrapyramidal symptoms (EPS) have been described with PDTC overdose and seem to result from an impairment of dopamine and glutamate neurotransmitter pathways (Vaccari *et al.*, 1999). It is of interest in this context that NO is implicated in dopamine disturbances and the induction of EPS evoked by neuroleptics (Harvey & Nel, 2003). However, toxicity of PDTC was not assessed in the present study. The slight decrease of PDTC-induced nitrite production in unstressed animals described in the present study is most likely attributed to the inhibitory effect of PDTC on the activation of NF $\kappa\beta$, which is absent under normal conditions, but only is expressed in inflammation, such as after TDS-stress. Thus, Figure 5-5 indicates that PDTC significantly decreased hippocampal NO_x levels after exposure of animals to TDS-stress. Since PDTC inhibits iNOS transcription/expression via NF $\kappa\beta$ (Madrigal *et al.*, 2001) as well as decreases NOx accumulation (Müllner *et al.*, 2002), this suggests that overproduction of NO in the hippocampus following TDS-stress is the result of iNOS induction and not activation of nNOS, consistent with the findings of Harvey *et al.* (2004a) and Madrigal *et al.* (2001; 2003).

That PDTC also suppressed TDS induced cGMP, though not significantly, is of interest, suggesting that GC-cGMP activation may also ensue from iNOS-mediated NO, not just nNOS as is more traditionally accepted. However, the transcription factor, NF $\kappa\beta$, is also recognized as a key mediator of physiological and pathological plasticity in the CNS. Ionotropic glutamate receptor stimulation and the subsequent influx of Ca^{2+} into the cell, potently triggers NF $\kappa\beta$ activation (Burr & Morris, 2002), suggesting that PDTC may also be targeting an NMDA-nNOS driven event. Indeed, activation of constitutive NOS also follows acute stress (De Oliveira *et al.*, 2000), while iNOS appears to play a more prominent role during chronic stress (Homayoun *et al.*, 2002). Oxidative stress and elevation of Ca^{2+} levels are also particularly important inducers of NF $\kappa\beta$ activation thus leading to iNOS stimulation (Mattson, 2001). Indeed, both iNOS and NMDA receptors have been implicated in TDS-stress (Harvey *et al.*, 2004a).

Since PTSD has its origin in an initial traumatic event or acute stressor, its long-term development represents a form of chronic, repeated stress due to flashbacks and re-experiencing. Consequently, it may be of major significance to consider that nNOS underlies the early stress-related events, but that a gradual, sustained increase in iNOS activity follows, that persists indefinitely. Figure 5-10 suggests a down-regulation of iNOS by PDTC via actions on NF $\kappa\beta$, but also leading to a decrease in GC activation and a subsequent decrease in cGMP production. Together, this suggests a putative role of iNOS and nNOS in stress-related disorders such as PTSD.

The glutamate-NMDA receptor occupies a central role in the stress response, in the process of LTP and memory (Tsien *et al.*, 1996) and more recently has been implicated in PTSD and its treatment (Heresco-Levy *et al.*, 2002). In this study, the role of NMDA receptor activation in TDS-stress was explored using the selective NMDA receptor antagonist, memantine. Memantine-hydrochloride is approved for patients with moderate to severe Alzheimer's disease (AD), while its efficacy in AD is thought to be due to block of glutamate receptors and decreasing the excitotoxic effects related to abnormal glutamate signalling (Glasko, 2003). Its mechanism of action is distinct compared to other approved Alzheimer's treatments (Stovell, 2003). This low-affinity, non-competitive, voltage-dependent NMDA receptor antagonist has also been implicated in and associated with behavioural improvement in patients with various neuropsychiatric disorders, including mood and motor disorders such as depression and Parkinson's disease, respectively (Gortelmeyer & Erbler, 1992).

Under control conditions, memantine decreased NO metabolites, although not significantly (Figure 5-14). Since memantine is a NMDA antagonist, and that NO/GC activation are directly linked to NMDA receptor activation, it would be expected that both NO_x and cGMP levels should be suppressed. However, memantine caused an increase in cGMP levels under non-stressed conditions (Figure 5-15), opposite to that which would be expected. NMDA antagonism via other drugs such as ketamine and phencyclidine has been reported to increase glutamatergic transmission through non-NMDA receptor mechanisms (i.e. AMPA and kainate receptors) (Sanacora *et al.*, 2003). This acute increase in glutamate transmission may contribute to the observed elevated cGMP levels.

Earlier studies have found that TDS-stress evokes a significant decrease in NMDA receptor density (Harvey *et al.*, 2004a). In this study, a sub-chronic challenge with memantine did not significantly modify the hippocampal NO_x response to TDS-stress (Figure 5-7). To be maximally effective, a receptor antagonist requires sufficient quantity of that specific receptor if it is going to be effective in attenuating the disease process. It has been suggested that this decrease in NMDA receptor number is linked to a reactive attempt of the brain to limit excess NMDA receptor activation that follows severe stress (Harvey *et al.*, 2004a; Oosthuizen *et al.*, 2005). This natural physiological response in the face of severe trauma may limit the use of NMDA antagonists, such as memantine, especially at a time point distal to the traumatic event because of the down-regulated receptors. However, since glutamate release that occurs immediately after stress may have long-term implications, memantine may be of use in the immediate aftermath of trauma. In other words, since TDS-stress emphasizes repeated trauma over time, it is very likely that memantine may not be effective much later after the initial traumatic event, as emphasized in this study. Since NO is able to down-regulate its own synthesis both directly on NOS (Griscavage *et al.*, 1995) and via down-regulation of the NMDA receptor (Zanelli *et al.*, 2002), the data revealed in Figure 5-7 may reflect NO-mediated down-regulation of NMDA receptors in the hippocampus following TDS-stress. Indeed, TDS-stress has been found to evoke a down-regulation of NMDA receptors in rat hippocampus (Harvey *et al.*, 2004a). Furthermore, it has also been suggested that NO may decrease NMDA-dependent effects by promoting oxidation of a modulatory site of the receptor protein (Lei *et al.*, 1992). These actions imply down-regulated NMDA receptors in the aftermath of stress. This receptor state will reduce the response to memantine.

In Figure 5-12 however, memantine still was able to significantly decrease the increased cGMP response to TDS-stress. This paradoxical effect on two points of the same pathway is of interest, and may reflect the pharmacology of memantine. Memantine is a use-dependent NMDA receptor blocker (Sanacora *et al.*, 2003), implying that under normal conditions, it will not block the NMDA channel completely, still allowing normal Ca^{2+} flux to occur. However, in pathological conditions, such as stress, where there is excess NMDA channel opening, it effectively blocks the channel. It is therefore of interest that under both normal and TDS conditions, memantine did not alter NO_x levels, but increased cGMP under basal conditions, yet blocked TDS-induced cGMP accumulation in the stressed animals. Thus, cGMP may have a more important role than NO in the TDS-stress response and in the

action of memantine. Nevertheless, the data are emphatic that an action on cGMP is evident, both as a target of stress and as a possible therapeutic target. This is further emphasized in the sildenafil studies described below. This, together with the data describing a less than satisfactory response to memantine under conditions of decreased regulated NMDA receptors, is supportive of an important place for targeting the sub-cellular pathways activated by NMDA receptors, but particularly GC-cGMP, in the aftermath of severe stress. This may represent a novel therapeutic strategy in trauma victims to prevent the long-term development of neuropathology associated with PTSD.

Activation of either ionotropic or metabotropic glutamate receptors results in an increase in NO production, possibly involving NOS-dependent and NOS-independent pathways (Yamada & Nabeshima, 1997). In the present study, treatment with the selective nNOS inhibitor, 7-NINA, on its own resulted in a slight but insignificant decrease in NO_x levels (Figure 5-14). However, 7-NINA significantly blocked the increase in NO_x evoked by TDS (Figure 5-6). Another selective nNOS inhibitor, 1-(2-trifluoromethylphenyl)-imidazole (TRIM) also decreases nNOS in areas related to stress reactions and anxiety (De Oliveira *et al.*, 2000; Volke *et al.*, 2003). Furthermore, Figure 5-11 also indicates that 7-NINA significantly decreased the enhanced cGMP response to TDS-stress. Since nNOS is directly linked to GC-cGMP, it seems logical that inhibition of nNOS will subsequently lead to diminished cGMP levels in the NO-cGMP cascade. These data provide emphatic proof of the prominent involvement of nNOS in TDS-stress, and is in agreement with earlier studies using restraint stress (De Oliveira *et al.*, 2000).

Figure 5-15, however, displays a significant increase in cGMP accumulation after treatment with 7-NINA. Synthesis of NO by nNOS is under regulation of the NMDA receptor, while NO can also regulate the NMDA receptor by down-regulating the NMDA receptor in a negative feedback loop (McCaslin & Oh, 1995). Of great importance is that NO can also be produced non-enzymatically from nitrite at low pH under reducing conditions (Aktin, 2004) resulting in the stimulation of GC and the production of cGMP. It can therefore be concluded that, in the presence of potent nNOS inhibition, NO production may still be maintained via a non-enzymatic mechanism, under reducing conditions, that leads to the production of its oxidative metabolites nitrite and nitrate (NOx), and activation of GC (Figure 5-15).

In the final drug challenge study, the selective and potent inhibitor of cGMP-specific PDE-5, sildenafil, was found to significantly increase cGMP in the hippocampus of non-stressed rats when compared to basal values (Figure 5-15). These data is in agreement with earlier studies on sildenafil (Corbin *et al.*, 2004; Turko *et al.*, 1999). However, sildenafil did not alter NOx levels (Figure 5-14), inducing a slight suppression in NOx levels when compared to the control. This apparent paradox may reflect a feedback mechanism between NO and cGMP. Indeed, cGMP has been found to modulate glutamate release therefore affecting activation of the NMDA channel, possibly resulting in the decrease in NO production observed in this study (Sistiaga *et al.*, 1997). Increases in cGMP levels in nerve terminals induce a depression of glutamate release by a mechanism involving the activation of a cGMP-dependent kinase (Sistiaga *et al.*, 1997), or may be mediated by the release of endogeneous adenosine (Sistiaga *et al.*, 1997).

Of major significance in this study, however, was that during stress, sildenafil was found to significantly bolster the stress-induced activation of NOx accumulation (Figure 5-8) as well as increase cGMP accumulation, although not significantly (Figure 5-13) in the hippocampus of stressed rats, in keeping with its actions on cGMP under control conditions. Sildenafil crosses the blood-brain barrier (BBB) and is thereby capable of exerting various neurologic, emotional, or psychological effects in human subjects (Milman & Arnold, 2002). Recent studies by Volke and colleagues (2003) and Kurt and colleagues (2004) have demonstrated that sildenafil has anxiogenic actions in rodents, while post-marketing surveillance studies (Milman & Arnold, 2002) have associated sildenafil with increased aggressive behaviour, a cardinal symptom of PTSD (McIntosh, 1999). These studies, as well as the current study, implicate these actions on sildenafil's influence on the NO-cGMP signalling pathway and its ability to enhance intracellular cGMP concentrations.

PDE-5 enzymes are present in particularly high concentrations in the hippocampus, cerebral cortex and basal ganglia (Garthwaite & Boulton, 1995) where they modulate the concentrations of cGMP. Elevations in cGMP may underlie the incidence of aggressive behaviour (Nelson & Chiavegatto, 2001), while Prickaerts *et al.* (2002; 2004) have also argued that inhibition of PDE-5 and increases in cGMP in the dorsal hippocampus underlies the improvement in object recognition memory observed with sildenafil. The results presented in this study provide the first data in an animal model of PTSD that sildenafil may enhance stress-induced responses. Since increased cGMP appears correlated with anxious states (Kurt *et al.*, 2004; Volke *et*

al., 2003), these data extends the role of cGMP from a pathological role in anxiety to a causal role in PTSD. It is also of interest that cGMP not only has a role in memory function (Prickaerts *et al.* 2002; 2004) but also is an important mediator of LTP and sensitisation (Boxall & Garthwaite, 1996) purported to be involved in the dysfunctional memory evident in PTSD (Oosthuizen *et al.*, 2005).

The present study has therefore brought a definitive causal association between TDS-stress with NO-cGMP, although further studies are needed to delineate the respective roles of nNOS and iNOS in the stress response. The evidence that increased cGMP is a protagonist of the stress response is an important observation, and further neuro-endocrine and behavioural studies are recommended. The involvement of NO-cGMP has great importance in explaining some of the most important behavioural and neuropathological characteristics of PTSD, but especially cognitive and memory disturbances, anxiety and hippocampal degeneration. Moreover, this may have important implications for pharmacological intervention and the treatment of acute stress and PTSD, but also in preventing the long-term development of PTSD after a severe traumatic event.

Chapter 7 Conclusion

Severe stress can precipitate lasting neuroendocrine and behavioural changes that can significantly affect functioning. PTSD is a debilitating anxiety disorder that develops after an individual has experienced or witnessed a severe, traumatic event. A lack of effective treatment and insufficient knowledge about the neurobiology and pathophysiology underlying PTSD, emphasises the fact that further pharmacological and behavioural studies on PTSD are needed.

Using time-dependent sensitisation (TDS), a putative animal model of PTSD that emphasises repeated trauma, a distinct causal role for NO was observed. Pharmacological studies designed to assess the role of the NO-cGMP pathway in TDS-stress, concur that stress activates the glutamate-NOS-cGMP pathway that is detectable at various levels of the NO-signalling cascade.

The study has provided convincing evidence that:

- TDS-stress significantly increases hippocampal NOS activity, as well as significantly raises NO_x and cGMP levels in animals subjected to stress.

- The observed increase in **NO_x** levels were significantly blocked by treatment with either the NF$\kappa\beta$ -inhibitor, PDTC or the nNOS inhibitor, 7-NINA, while the NMDA receptor antagonist, memantine, was without effect. Sildenafil, the PDE-V inhibitor, significantly augmented the stress-induced increase in NO_x.

- Both 7-NINA and memantine significantly blocked the TDS-induced increase in **cGMP**, while PDTC attenuated this response, but not significantly. Sildenafil, however, increased the cGMP levels although not significanly.

Thus, both iNOS and nNOS activation underlies TDS-stress, while NMDA receptor involvement may be less critical during chronic stress. Activation of GC-cGMP appears to be a potent protagonist of the response to stress.

A definite association between TDS-stress and the NO-cGMP pathway is therefore evident. The involvement of NO-cGMP has great importance in explaining the neuropathological abnormalities characteristic of PTSD, such as anxiety and hippocampal degeneration, but also implicates the NOS-cGMP pathway as novel targets for pharmacological interventions in the treatment of PTSD.

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