A pharmacokinetic – pharmacodynamic relationship study between GABA-ergic drugs and anxiety levels in an animal model of PTSD.

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B.Pharm

Dissertation submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTAE

in the

School of Pharmacy (Pharmacology)

at the

North-West University

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Potchefstroom
2005

Abstract

Posttraumatic stress disorder (PTSD) is classified as an anxiety disorder and the characteristic symptoms (re-experiencing, avoidance as well as numbing of general responsiveness and hyperarousal) of this disorder develop in response to a traumatic event. The disorder is characterised by hypothalamic-pituitary-adrenal (HPA) axis abnormalities linked with changes in cortisol moreover, the hippocampus and cortex also play a role in the neurobiology. With regard to the neurochemistry of this disorder it is known that gamma amino butyric acid (GABA) is involved however, the precise role of GABA in PTSD and how stress changes GABA concentrations in the brain are still not fully understood. Another aspect regarding PTSD that has not been clearly defined is the treatment of PTSD. Classic anxiolytics such as diazepam is expected to relieve the anxiety linked with PTSD. Studies with this group of drugs have however not produced the concrete evidence needed to establish it as a treatment of choice for PTSD and subsequently other classes of drugs have been investigated as possible treatment options for PTSD. Among these is lamotrigine, which in a clinical study was found to be effective in alleviating symptoms of PTSD. Moreover, a possible pharmacokinetic-pharmacodynamic relationship for each of these drugs has also not been elucidated.

In order to elude on some of these uncertainties, an animal model of PTSD, time dependent sensitisation (TDS), was used. GABA levels in the rat hippocampus and frontal cortex were determined at two different time intervals following the TDS procedure (1 day and 7 days post re-stress). High performance liquid chromatography (HPLC) with electrochemical (EC) detection was used to determine gamma amino butyric acid (GABA) concentrations. To investigate the possible anxiolytic effects of diazepam and lamotrigine in this model, as well as a possible pharmacokinetic-pharmacodynamic relationship for each drug, pharmacokinetic profiles for both drugs were established in order to find the times of peak and trough levels of each drug. Blood samples were collected at different time intervals after drug administration either from the tail vein of rats (lamotrigine) or directly from the heart (diazepam). Subsequently, drug concentrations at each time interval were determined by means of HPLC with ultraviolet (UV) detection. The behaviour of rats was analysed using the elevated plus-maze (EPM) at peak or trough concentrations of the drugs and this was performed after either acute administration of the drug, or after a 14 day chronic treatment regime.

GABA levels in the hippocampus were not found to change statistically significantly in response to stress at either 1 day or 7 days post re-stress. In the frontal cortex, however, GABA levels

increased in response to stress at 1 day post re-stress, with a statistically insignificant, but strong trend towards an increase, at 7 days post re-stress. With regard to the pharmacokinetic profiles, the peak concentration of diazepam was found to occur at 60 minutes, with lamotrigine's peak at 120 minutes. The behavioural studies indicated that acute treatment with diazepam 3 mg/kg resulted in a statistically significant increase in both ratio open arm entries and ratio time spent in the open arms at peak level of the drug. After acute treatment with diazepam 3 mg/kg a statistically significant decrease in ratio time spent in open arms was also found when the ratio time spent in open arms at peak level of the drug and the ratio time spent in open arms at trough level of the drug was compared. In response to chronic treatment with diazepam 3 mg/kg for 14 days, test animals exhibited an increase in the ratio open arm entries at trough level of the drug, with a statistically insignificant yet definite trend towards an increase at peak level. Acute treatment with lamotrigine 10 mg/kg resulted in no statistically significant change in EPM parameters. In response to chronic treatment, however, a statistically significant increase was found in ratio time spent in open arms at peak level of the drug, with a statistically insignificant trend towards an increase at trough level.

From the results of this study, we may therefore conclude that GABA-levels in the brain are definitely affected, but in different ways, following TDS-stress. A pharmacokinetic-pharmacodynamic relationship between the drugs' levels and aversive behaviour could also be established. Furthermore it appears that more sustained anxiolytic effects are evident following chronic treatment with both drugs than with acute administration of these drugs.

Key words: Posttraumatic stress disorder (PTSD), time dependent sensitisation (TDS), elevated plus-maze (EPM), hippocampus, frontal cortex, gamma amino butyric acid (GABA), diazepam, lamotrigine, rats.

Opsomming:

Posttraumatiese stres sindroom (PTSS) word geklassifiseer as 'n angsversteuring en word gekenmerk deur simptome (aanhoudende herlewing van die trauma, vermyding van herinneringe verbonde aan die trauma, verhoogde outonomiese opwekking) wat ontwikkel in reaksie op die trauma. Die sindroom word gekenmerk deur HPA-as abnormaliteite wat verband hou met veranderinge in kortisol vlakke; verder speel die hippokampus en frontale korteks ook 'n rol in die neurobiologie van PTSS. Met betrekking tot die neurochemie is dit bekend dat gamma-aminobottersuur (GABA) betrokke is, alhoewel die spesifieke rol van GABA en die invloed van stres op GABA vlakke nog steeds nie ten volle begryp word nie. 'n Volgende aspek van PTSS wat ook nog nie ten volle opgeklaar is nie, is die behandeling van die toestand. Daar sou verwag kon word dat bekende angsiolitikums soos diasepam verligting sou bring van angs wat ook geassosieer word met PTSS. Studies wat met hierdie groep middels gedoen is, het egter nog nie konkrete bewyse gelewer wat hierdie groep as 'n behandelingskeuse vir PTSS sal vestig nie. Ander groepe geneesmiddels word derhalwe getoets as moontlike behandeling vir PTSS waaronder lamotrigine, wat simptome van PTSS effektief verlig het in 'n kliniese studie. Hiermee saam is 'n moontlike farmakokinetiese-farmakodinamiese interaksie vir elk van hierdie geneesmiddels ook nog nie vasgestel nie.

Met die oog daarop om nuwe lig te werp op sekere van die bogenoemde onsekerhede, is 'n dieremodel van PTSS, tydafhanklike sensitisasie (TAS), gebruik. GABA vlakke is bepaal in die rot hippokampus en frontale korteks op 2 verskillende tye na die TAS prosedure (1 dag en 7 dae na herblootstelling aan stres). Hoëdigtheidsvloestofchromatografie met elektrochemiese deteksie is gebruik om die GABA konsentrasies te bepaal. Om die moontlike angsiolitiese effekte van diasepam en lamotrigine in hierdie dieremodel te ondersoek, asook 'n moontlike farmakokinetiese-farmakodinamiese interaksie vir beide geneesmiddels, is farmakokinetiese profiele van beide geneesmiddels vasgestel om die tye van piek- en trogkonsentrasies van elke geneesmiddel te bepaal. Bloed monsters is geneem uit die stertaar van rotte (lamotrigine) of direk uit die hart (diasepam) op verskillende tye na geneesmiddel toediening. Hieropvolgend is die geneesmiddelkonsentrasies by elke tydinterval bepaal deur hoëdigtheidsvloestofchromatografie met ultraviolet deteksie. Die gedrag van die rotte is geanaliseer deur middel van die "Elevated Plus-Maze (EPM)" by piek of trog vlakke van die geneesmiddels en hierdie prosedure is uitgevoer na akute toediening van die geneesmiddel of na 'n kroniese behandeling van 14 dae.

GABA vlakke in die hippokampus het nie statisties betekenisvol verander in reaksie op stres op 1 dag of 7 dae na herblootstelling aan stres nie. In die frontale korteks egter, het GABA vlakke verhoog in reaksie op stres op 1 dag na die herblootstelling aan stres, met 'n statisties onbetekenisvolle, maar relevante neiging tot 'n verhoging op 7 dae na herblootstelling aan stres. Wat die farmakokinetiese profiele betref is bevind dat die piek konsentrasie van diasepam voorkom by 60 minute en lamotrigine se piek by 120 minute. Die gedragstudies dui aan dat akute behandeling met diasepam 3 mg/kg gelei het tot 'n statisties betekenisvolle verhoging in beide die verhouding oop arm toetredes en verhouding tyd spandeer in die oop arms by piek vlakke van die geneesmiddel. Na akute behandeling met diasepam 3 mg/kg is 'n statisties betekenisvolle verlaging in verhouding tyd spandeer in oop arms ook gevind indien die verhouding tyd spandeer in oop arms by piek vlak van die geneesmiddel vergelyk is met verhouding tyd spandeer in oop arms by trog vlak van die geneesmiddel. In reaksie op chroniese behandeling met diasepam 3 mg/kg vir 14 dae het rotte 'n verhoging getoon in verhouding oop arm toetredes by trog vlakke van die geneesmiddel en ook 'n statisties onbetekenisvolle dog definitiewe neiging tot 'n verhoging by piek vlakke. Akute behandeling met lamotrigine 10 mg/kg het tot geen statisties betekenisvolle veranderinge in EPM parameters gelei nie. In reaksie op chroniese behandeling, egter, is 'n statisties betekenisvolle verhoging gevind in verhouding tyd spandeer in oop arms by piek vlak van die geneesmiddel, asook 'n statisties onbetekenisvolle neiging tot 'n verhoging by trog vlak van die geneesmiddel.

Uit die resultate van hierdie studie kan ons aflei dat GABA vlakke in die brein definitief deur TAS stress beinvloed word, maar op verskillende maniere. 'n Farmakokinetiese-farmakodinamiese verwantskap tussen geneesmiddel vlakke en angstige gedrag kon ook vasgestel word. Verder wil dit ook voorkom asof 'n meer langdurige angsiolitiese effek voorkom na chroniese behandeling met beide geneesmiddels as na akute toediening met hierdie geneesmiddels.

Sleutelwoorde: Posttraumatiese stress sindroom (PTSS), tyd afhanklike sensitisasie (TAS), elvated plus maze (EPM), hippokampus, frontale korteks, gamma amino botter suur (GABA), diasepam, lamotrigine, rotte.

Acknowledgements

Ek wil begin deur dankie te se vir God. Hy het my nooit alleen gelaat deur hierdie hele studie nie en ek wil Hom ook dank vir die geleentheid om hierdie MSc studie te kon aanpak en voltooi.

Die afgelope twee jaar was 'n verrykende ervaring wat ek in die toekoms altyd met 'n glimlag sal onthou. Dit sou 'n veel armer ervaring gewees het sonder al die mense wat betrokke was en daarom wil ek die volgende mense graag bedank:

❖ My wonderlike studie-leiers Dr Malie Rheeders en Prof Linda Brand.
Baie dankie vir julle onbaatsugtige hulp en ondersteuning reg deur die afgelope twee jaar; julle het die fondasie gelë vir my as navorser. Geen M-student kan vir twee beter studie-leiers vra nie!

● Izelle Grobler en Anita Pretorius.
Baie dankie vir julle vriendskap deur die afgelope twee jaar, dit was 'n voorreg om hierdie spesiale ervaring in my lewe met julle te kon deel.

● Die hele Departement Farmakologie.
Dit was 'n voorreg om in so 'n heerlike werksomgewing te kon werk aan hierdie studie. Baie dankie vir al die vriendelikheid en ondersteuning van almal af en dat julle ons reg van die begin af so welkom en deel van die departement laat voel het.

● Prof Brian Harvey vir sy waardevolle insette en hulp.

❖ Francois Viljoen.
Baie dankie vir al jou hulp spesifiek met die HPLC werk, dit was van onskatbare waarde!

❖ Dr Jan Du Preez vir sy waardevolle insette en hulp met die HPLC bepalings.

❖ Cor Bester en die personeel van die Proefdiesentrum.
Baie dankie vir al die hulp met die rotjies, ek sou dit nooit alleen kon doen nie.

● Prof Faans Steyn vir sy statistiese verwerking van die resultate en al sy hulp.

● Prof Breytenbach vir die taalversorging van hierdie verhandeling

● Ek wil ook graag dankie sê vir Prof Oliver, Prof Brand en die Skool vir Farmasie wat ons die geleentheid gegee het om die SAPS kongres by te woon. Die ervaring het my wetenskaplike horisonne verbreed en dit was geweldig leersaam. Dit was 'n sonderlinge ervaring wat ek om verskeie redes nooit sal vergeet nie!

● My pa, Johan en my ma, Marie.

Baie dankie vir julle onbaatsugtige liefde en ondersteuning en die geleentheid wat julle my gebied het om my studies verder te voer en my horisonne te verbreed.

❖ Al my vriende buite die Departement.

Baie dankie vir julle ondersteuning en al die belangstelling wat julle getoon het, julle het 'n groot aandeel aan die suksesvolle afhandeling van my MSc.

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Jacolene Myburgh, Malie Rheeders, Linda Brand & Brian H Harvey

"The relationship between GABA levels and anxiety as determined by an animal model of posttraumatic stress disorder." South African Pharmacology Society (SAPS) Congress. *13-16 September 2005, Cape Town, South Africa.*

**Second runner-up in the Young Scientist Competition.

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Abbreviations

5-HT	:	Serotonin
ACTH	:	Adrenocorticotropin releasing hormone
$AUC_{(0-t)}$	:	Area under curve, zero until last measured time
$AUC_{(0-\infty)}$	:	Area under curve, zero until infinity
C_{max}	:	Maximum concentration
CR	:	Conditioned response
CRH	:	Corticotropin releasing hormone
CNS	:	Central nervous system
CRF	:	Corticotropin-releasing factor
CS	:	Conditioned stimulus
CSF	:	Cerebrospinal fluid
DA	:	Dopamine
DLPFC	:	Dorsolateral prefrontal cortex
DRN	:	Dorsal raphe nucleus
EC	:	Electrochemical
EPM	:	Elevated plus maze
GABA	:	Gamma aminobutyric acid
GAD	:	Glutamate decarboxylase
GR	:	Glucocorticoid receptor
HPA axis	:	Hypothalamic-pituitary-adrenal axis
HPLC	:	High performance liquid chromatography
iNOS	:	Inducible nitric oxide synthase
k_e	:	Elimination constant
MAOI	:	Monoamine oxidase inhibitor
MPFC	:	Medial prefrontal cortex
MR	:	Mineralocorticoid receptor
MWM	:	Morris Water Maze
NA	:	Noradrenaline
NMDA	:	*N*-methyl-D-aspartate
NO	:	Nitric oxide
NOS	:	Nitric oxide synthase
NPY	:	Neuropeptide Y
OMPFC	:	Orbital and medial prefrontal cortex

PAG	:	Peri-aquaductal-grey
PFC	:	Prefrontal cortex
PVN	:	Paraventricular nucleus
PTSD	:	Posttraumatic stress disorder
SRI	:	Serotonin reuptake inhibitor
SSRI	:	Selective serotonin reuptake inhibitor
$\mathbf{T_{1/2}}$	:	Half-life
$\mathbf{T_{max}}$	:	Time of maximum concentration
TCA	:	Tricyclic antidepressant
TDS	:	Time-dependent sensitization
UR	:	Unconditioned response
US	:	Unconditioned stimulus
UV	:	Ultra violet

Introduction

1. *Background:*

Posttraumatic stress disorder (PTSD) is an anxiety disorder that develops in response to a traumatic ordeal that involves actual or threatened death or serious injury to one self or a loved one (National Institute of Mental Health, 2001). According to the Diagnostic and Statistical Manual of Mental disorders, 4th edition (DSM-IV) (APA, 1994), the essential feature of PTSD is the development of characteristic symptoms after the trauma. These symptoms include persistent re-experiencing of the traumatic event, persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness and persistent symptoms of increased arousal. PTSD does not necessarily develop directly after the traumatic experience; it can develop up to more than 6 months afterwards.

If we consider the vast range of the symptoms of PTSD, it is clear that various neurobiological systems must be involved in PTSD (Albucher & Liberzon, 2002). The hypothalamic-pituitary-adrenal axis (HPA axis) consists of the hypothalamus, the pituitary as well as the adrenal gland and controls the body's reaction to stress. This axis manages the stress reaction by receiving and interpreting information from areas of the brain such as the amygdala and hippocampus and also from the autonomic nervous system (Shea *et al.*, 2004). The amygdala is the main coordinator of the fear response and information is sent to the amygdala from several brain regions, which include the medial cortex, the hippocampus and the cortico-striato-thalamic circuits. Hippocampal dysfunction may be linked to the overgeneralization of fear responding, an important feature of the anxiety disorders (Kent *et al.*, 2002). The hippocampus is a component of the limbic stress pathway, along with the cortex, septum and amygdala. The volume of the hippocampus may be diminished in PTSD patients, possibly having a considerable influence on the pathophysiology of PTSD (Sala *et al.*, 2004).

Memory functioning is a very important aspect of the psychopathology of PTSD and moreover, the intrusive recall of memories of the trauma is an important characteristic of PTSD. Cognitive difficulties that are not linked to the traumatic event, have been seen in PTSD studies. Difficulties involve learning and memory abilities linked to working memory and connected to executive function. Among these difficulties are the reaction to novelty and the monitoring and

regulation of memories. Normal memory systems are crucial to continuous management of information, which is of utmost importance to normal living (Clark *et al.*, 2003).

Recent experimental results link various neuroanatomical circuits, neurotransmitter systems and neuronal mechanisms to PTSD pathophysiology. Central catecholamines, serotonin and extrahypothalamic corticotropin-releasing factor (CRF) are all involved in stress response regulation, as well as with the regulation of fear, anger, arousal and aggression. These are all functional domains that are frequently seen to be problem areas for PTSD patients (Albucher & Liberzon, 2002). Neurochemically, PTSD involves the following neurotransmitters: noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (serotonin) (5-HT), glutamate and gamma amino butyric acid (GABA) (Newport & Nemeroff, 2000). The hypothalamic-pituitary-adrenal (HPA) axis plays a role in the pathogenesis of PTSD and GABA modulates this axis. GABAergic systems also influence the pathogenesis of anxiety, depression and insomnia; these are problems experienced by PTSD patients. These findings led to the hypothesis that GABAergic systems may be involved in the modulation of PTSD. Glutamate and GABA play an important part in the process of factual memory registration and in encoding emotional and fear memory. GABA activity in the amygdala is crucial for fear conditioning. It is seen from previous studies that an increase in GABAergic activity in the amygdala may decrease vulnerability to stress (Vaiva *et al.*, 2003).

The abovementioned neuroanatomical circuits, neurotransmitter systems and neuronal mechanisms are the targets in pharmacological interventions with the aim to ease PTSD symptoms and this led to the experimentation with several drugs and classes of drugs as possible treatment options for PTSD. Many of these drugs do have some ability to alleviate certain symptoms of PTSD, but a single drug has not been found that combats PTSD as a whole. When investigating the pharmacological treatment of PTSD, one should also take into account that mechanisms of kindling and sensitization have been proven to regulate mood and emotional memory (Albucher & Liberzon, 2002). Kindling can be described as an elevation in seizure activity when a sub threshold stimulus is applied repeatedly to certain brain structures (Goddard *et al.*, 1969) and have been observed in limbic structures such as the amygdala that may be involved in stress response, fear and potentially in PTSD symptoms. Thus, this prompts investigation into whether "anti-kindling" agents could be used to treat specific PTSD symptoms. Quite a few of the newer anticonvulsants have demonstrated anti-kindling properties. Only a few controlled trials have been published, up to date, on the use of anticonvulsants in the treatment of PTSD. Lamotrigine was compared to placebo in a double blind fashion in 15 patients for 12 weeks and the research team found that lamotrigine may be effective as a primary pharmacological treatment in combat and civilian PTSD, as well as having a high level

of effectiveness in re-experiencing, avoidance and numbing of general responsiveness (Hertzberg *et* al., 1999).

2. *Problem statement:*

From evidence in the literature, it is clear that GABA plays a very crucial role in the development of PTSD. It is consequently important to know how GABA levels are influenced in different areas of the brain by the development of PTSD and anxiety – does GABA diminish or are GABA levels elevated?

Current treatment for PTSD is not always effective and new treatment options need to be investigated. As already mentioned the kindling phenomenon has a very interesting link to PTSD and anti-kindling agents can be a viable prospect as treatment option for PTSD. It would be very beneficial to know if and how these drugs would influence anxiety as a symptom of PTSD. Moreover, the question also remains if there would be a correlation between the extent to which a drug would exert an anxiolytic effect and the different drug concentrations over time in the body – put in short, is there a relationship between the pharmacokinetics of the drug and the pharmacodynamic effect of the drug in a condition like PTSD?

3. *Study objectives:*

- The first aim of the study was to determine GABA levels at 2 different time intervals in the hippocampus and frontal cortex of male Sprague-Dawley rats in control as well as TDS-stressed animals.
- ❖ The second aim was to construct pharmacokinetic profiles for both diazepam and lamotrigine in the rats in order to investigate the possible relationship between the drugs' levels and aversive behaviour.
- Lamotrigine as a glutamate inhibitory anti-convulsant with anti-kindling properties and diazepam, a GABAergic anxiolytic benzodiazepine were investigated for their possible effects in the TDS animal model of PTSD following acute and chronic administration. Anxiolytic effects were determined by means of the elevated plus-maze (EPM) animal model.

4. *Project layout:*

- ❖ The determination of GABA levels in the hippocampus and frontal cortex of rats was done by dividing a group of 24 male Sprague-Dawley rats into 2 groups of 12 each, where the first group of 12 was stressed using the TDS model and the second group served as a control group that was not stressed at all. The day after re-stress the animals were decapitated and determination of the GABA concentrations was done by using a high performance liquid chromatography (HPLC) method with EC detection. Another group of 24 rats were treated in exactly the same manner, but were decapitated 7 days post re-stress and GABA levels were subsequently determined in the same way.
- ❖ To establish a pharmacokinetic profile of each of the drugs the procedure was as follows: for diazepam 0.5 ml blood samples were taken directly out of the heart of 10 rats with each rat's sample being collected at one specific time after drug administration. To establish the pharmacokinetic profile of lamotrigine, three 0.5ml blood samples were taken from the rats' tail veins at 3 different times during the time profile. The blood samples were analysed by a validated HPLC method with UV detection.
- ● For the behavioural study a total of 192 rats were used. All those rats were exposed to the time dependant sensitization (TDS) procedure followed by analysis of their behaviour on the EPM. 108 rats received acute doses of either lamotrigine (10 mg/kg) or diazepam (3 or 5 mg/kg) over a 24 hour period before exposure to the EPM, while 36 of those TDS-stressed rats received saline instead of the drug and served as controls. Another 36 of the 192 rats received daily doses of either lamotrigine (10 mg/kg) or diazepam (3 mg/kg) for a 2-week period following the TDS procedure and before exposure to the EPM to examine the chronic effects of the drugs. These rats' behaviour was also compared with that of a control group, comprising of 12 rats which received saline.

Chapter 1: PTSD: an Anxiety Disorder

1.1 Introduction

Anxiety is an emotion familiar to each and every one of us. It can be of positive use when it moves us to act when required, but in the case of an anxiety disorder, though, this normally positive emotion can cause exactly the opposite result. Sufferers from an anxiety disorder could feel anxious most of the time, with lack of any cause. These anxious feelings can be so uncomfortable that to avoid them, the patient will refrain from doing certain everyday activities; the patient may also experience sporadic episodes of anxiety that are so intense that they terrify and immobilize the patient (Mental Help Net, 2004). Anxiety can be described as an intricate feeling of apprehension, fear and worry frequently linked with pulmonary, cardiac and other physical sensations (Hsu, 2004).

Anxiety can be conceptualised dually as a state and as a trait. Trait anxiety can be described as the constant and enduring characteristic of the individual personality which shows how the individual interacts with their physical and social surroundings. State anxiety is when the anxiety occurs in a specific person at a specific time (Sandford *et al.*, 2000).

The DSM IV (APA, 1994), classifies several disorders as anxiety disorders and among these posttraumatic stress disorder is also found.

1.2 Posttraumatic stress disorder

The uniqueness of PTSD is captured in the significance of the traumatic stressor, in the absence of which a diagnosis of PTSD cannot be made (Friedman, 2003). PTSD used to be a diagnosis surrounded by debate. Currently, however, this disorder is widely acknowledged as a valid diagnostic entity, as well as accumulating a noteworthy database of neurobiological research (Newport & Nemeroff, 2000).

The following are the specific diagnostic criteria for PTSD as stipulated in the DSM-IV (APA, 1994):

(a) The individual has been exposed to a traumatic event in which both of the following were present:

- The individual experienced, witnessed or was confronted with an event or events that involved actual or threatened death or serious injury, or a threat to the physical integrity of him or herself or others.

- The individual's response involved intense fear, helplessness or horror. Note: In children, this may be expressed instead by disorganised or agitated behaviour.

(b) The traumatic event is persistently re-experienced in one or more of the following ways:

- Recurrent and intrusive distressing recollections of the event, including images, thoughts, or perceptions. Note: In young children repetitive play may occur in which themes or aspects of the trauma is expressed.

- Recurrent distressing dreams of the event. Note: In children, there may be frightening dreams without recognisable content.

- Acting or feeling as if the traumatic event were recurring (includes a sense of reliving the experience, illusions, hallucinations and dissociative flashback episodes, including those that occur on awakening or when intoxicated). Note: In young children, trauma-specific re-enactment may occur.

- Intense psychological distress at exposure to internal or external cues that symbolise or resemble an aspect of the traumatic event.

- Physiological reactivity on exposure to internal or external cues that symbolise or resemble an aspect of the traumatic event.

(c) Patient avoidance of stimuli associated with the trauma and numbing of general responsiveness (not present before the trauma), as indicated by three or more of the following:

- Efforts to avoid thoughts, feelings or conversations associated with the trauma

- Efforts to avoid activities, places or people that arouse recollections of the trauma

- Inability to recall an important aspect of the trauma

❖ Markedly diminished interest or participation in significant activities

❖ Feeling of detachment or estrangement from others

❖ Restricted range of affect, for example, the inability to experience loving feelings

❖ Sense of a foreshortened future, of example, the individual does not expect to have a career, children, a marriage, or a normal life span

(d) Persistent symptoms of increased arousal (not present before the trauma), as indicated by two or more of the following:

❖ Difficulty falling or staying asleep

❖ Irritability or outbursts of anger

- Difficulty concentrating

❖ Hypervigilance

❖ Exaggerated startle response

(e) Duration of the disturbance is at least a month.

(f) The disturbance causes clinically significant distress or impairment in social, occupational or other important areas of functioning.

Three clusters of PTSD symptoms can be identified, namely re-experiencing, avoidance and numbing of general responsiveness and thirdly hyperarousal, as specified in the DSM-IV (APA, 1994) and discussed above. Sometimes PTSD symptoms wane with time, but in some cases they remain present for years (APA, 2000).

The symptoms a trauma victim experiences directly post trauma are comparable to PTSD symptoms. Within the three symptom clusters of PTSD, it is found that intrusive recollections and increased arousal are mostly present rapidly post trauma. Most commonly avoidance will only precipitate later on and it is this cluster of symptoms that frequently undoubtedly confirms a diagnosis of PTSD (North, 2001).

Thoughts, memories, perceptions, images or dreams might all play a part in re-experiencing. The traumatic event unremittingly intrudes into awareness as a result of being triggered by external or internal and frequently unexceptional stimuli. This is an extremely taxing emotional response. In extreme cases the victim could lose track of time and place, known as dissociation. The mechanism of fear conditioning has been considered as a model for the re-experiencing symptoms of PTSD because of the link that exists between traumatic recall and unrelated stimuli and the fearful response that follows (Le Doux, 2000).

Studies on conditioned fear responses have most frequently been done on rodents. When an animal is exposed to a non-threatening stimulus, known as a conditioned stimulus (CS) and an aversive stimulus known as an unconditioned stimulus (US), the animal will start to develop a fear response (conditioned response, CR) in the case of exposure to the CS alone. If an animal is placed in the same surroundings to those where the experiment the animal was used in took place, a CR will also be observed. The two mentioned aspects of fear conditioning are known as "explicit cue" and "context" conditioning. Fear conditioning can set in rather quickly (Maren, 2001) and frequently only a once off experience of a CS-US is needed to provoke a conditioned response to stimuli that was perceived as neutral before. Fear conditioning can also be exceptionally persistent due to the CS-CR duo having the potential to be active indefinitely (Le Doux, 2000). Extinction occurs when after conditioning has set in, a CS continuously presented without a US and furthermore, in this process the CR is reduced. This is not a result of forgetting or memory erasure (Pearse & Bouton, 2001), but it happens when new non-aversive associations are formed that replace the previously formed fear-conditioned associations. These aversive conditioned associations are not erased and can potentially be reactivated if the patient is subjected to specific circumstances after extinction (Bouton, 2000).

Fear conditioning can help an individual to adapt in a life-threatening situation (Aardal-Eriksson *et al*, 2001). It helps by ensuring the best response to danger, keeping alert to danger and preventing the wavering of attention (Grillon, 2002).

Conditioned fear responses in PTSD are maladaptive and cause fear and apprehension. This is in contrast to the abovementioned. In PTSD, re-experiencing is caused by various trauma related, non-trauma related and possibly internal, ill defined stimuli. PTSD patients have a diminished capacity to differentiate between threat-related and non-related stimuli.

It is concluded that PTSD's re-experiencing symptoms are due to implementation of impaired and maladaptive fear conditioning-like mechanisms that are a response to extreme stress.

There are numerous corresponding mechanisms that could possibly explain the metamorphosis of adaptive fear conditioning into uncontrollable re-experiencing in PTSD:

(a) emotional-fear memories are more lucidly stored and easier to recall
(b) an unconditioned response (UR) can continue in the absence of a US
(c) here the ability to integrate context and content related information into one coherent stimulus is lost
(d) no decline in conditioned response to general stimuli is experienced
(e) no extinction is present even with the lack of stimulus reinforcement
(f) the experience of inescapable stress and the following learned helplessness boosts fear conditioning (Bonne *et al*, 2004).

This is elaborated on in **Figure 1-1** and **Table 1-1**.

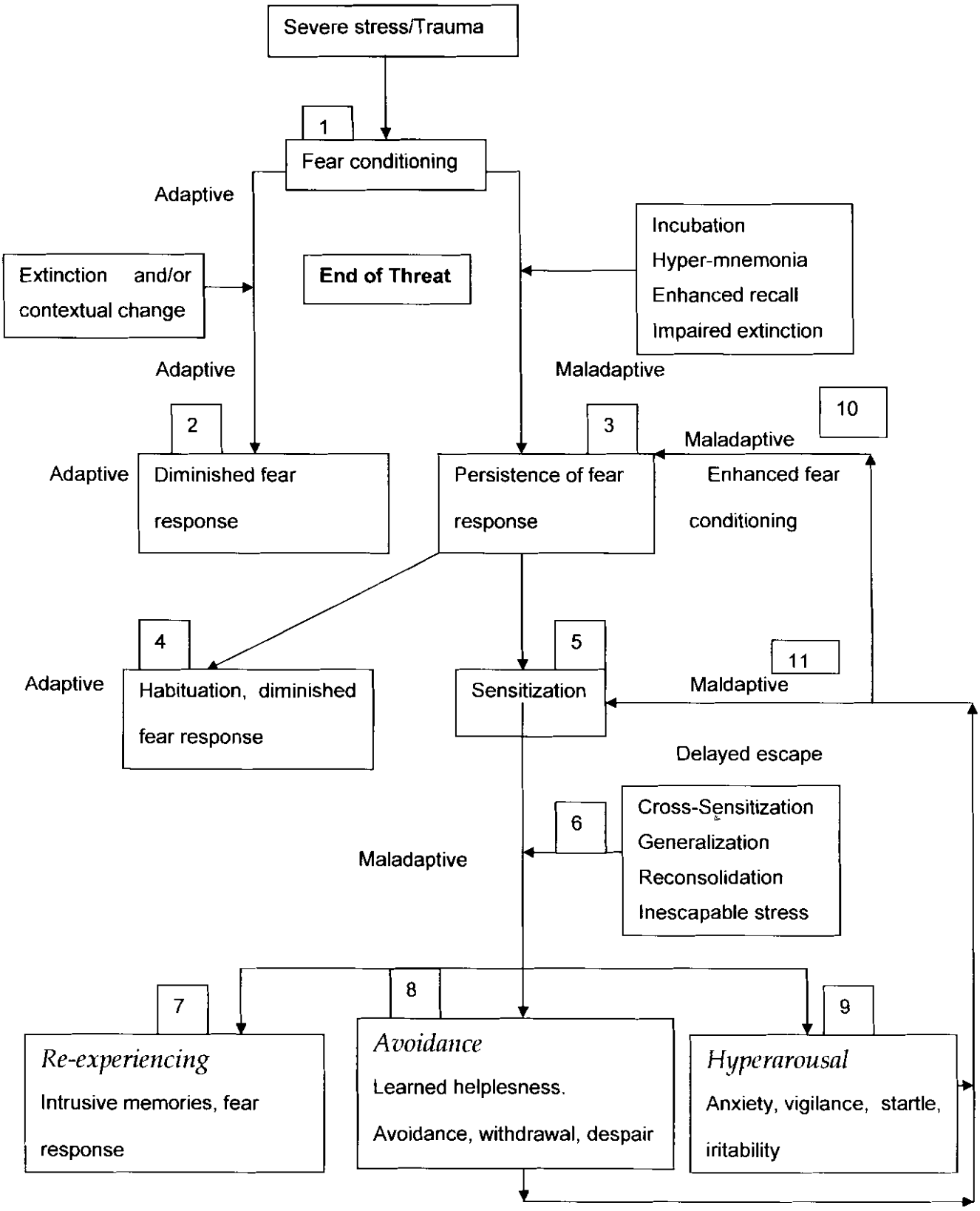


Figure 1-1: Adaptive and pathological responses to a severe stressor (Bonne et al., 2004).

Table 1-1: Neurobiological mechanisms and clinical symptom clusters in PTSD (Bonne et al., 2004).

Cluster	**Clinical symptom**	**Psychophysiological model**	**Neurocircuitry**	**Neurochemistry**	**Impairment in PTSD**
Re-experiencing	Intrusive recollections, thoughts, dreams; re-experiencing; intense psychological distress & fear	Maladaptive fear conditioning/ delayed extinction. Incubation. Increased re-consolidation. Impaired occasion setting	Amygdala, thalamus, hippocampus, anterior cingulate and prefrontal cortex, locus coeruleus, primary somatosensory cortices, insula	Corticotropin releasing hormone (CRH), cortisol Mineralocorticoid receptor, catecholamines, glutamate, GABA,	Fear conditioning persists despite absence of threat. Extinction is delayed, due to the intrinsic problem in this mechanism due to hyper mnemonic encoding and/or enhanced recall
Avoidance	Avoidance of intrapsychic or environmental recollections of trauma. Social withdrawal, detachment and diminished interest. Sense of foreshortened future	Inescapable stress – learned helplessness	Dorsal raphe nucleus, amygdala, thalamus, hippocampus, prefrontal cortex, nucleus accumbens, ventral tegmental area, periaquaductal grey	Serotonin, dopamine, CRH glutamate, GABA	Distresfull, un-Avoidable, repeated intrusive recolloctions become an "inescapable" stressor, leading to a "learned helplessness" like condition
Arousal	Anxiety,	Delayed/absent	Locus coeru-	Norepinephrine,	Due to

	insom-nia, lack of concentration, irritability, hyper-vigilance, startle	habituation. Sensitization. Cross sensitiza-tion. Generalization	leus, bed nucleus stria terminalis, amygdala, hippocampus, nucleus accum-bens, paraven-tricular hypothalamic nuclei, ventral tegmental area, periaquaductal grey	neuropeptide – Y- Glutamate, GABA, cortisol, dehydroepiandro-sterone, serotonin	generalization And cross sensitization of threatening contextual stimuli, unsafe environment expands, all secure havens are abolished, leading to unremitting anxiety

Avoidance symptoms and depression like symptoms are the two main types of symptoms that make up the second symptom cluster of PTSD. Avoidance symptoms can be either emotional avoidance, such as the avoidance of thoughts, feelings or conversations associated with the trauma or physical avoidance, such as the avoidance of activities and people or places that can trigger memories of the traumatic event. These symptoms bare a similarity to specific animal behaviour after the experience of severe stress described as "learned helplessness" and have been observed before by different researchers (Maier 2001). Because of the vast variation in the definition of the stress that causes learned helplessness, such stress was classified as inescapable stress. Inescapable stress with resulting learned helplessness has been used as an animal model for depression, anxiety as well as PTSD and was found to be an excellent description of this particular symptom cluster of PTSD (Bonne *et al,* 2004).

Symptoms of persistent anxiety and motor hyper-responsivity encompass the cluster of PTSD symptoms known as the increased arousal and persistent anxiety cluster. Common deficiencies here include difficulties in sleep and concentration, irritability and the patient being hyper-vigilant and "jumpy". Sufferers also lose their essential feeling of safety (Janoff-Bulman, 1992). As previously mentioned, the ability to distinguish between safe and unsafe is lost in PTSD and this failure become very much apparent when it is noted how a PTSD sufferer will generalize all stimuli with the effect that the fear response is triggered. Simultaneously to this an increase of the threatening context occurs which can be so great that almost all environments become unsafe to the PTSD sufferer with the result that danger becomes impending and erratic. The

outcome of this is that the PTSD patient is in a constant condition of anticipatory anxiety and this elevates the patient's vulnerability to future exposure to trauma (Bonne *et al*, 2004).

The brain has the ability to react to stress rather quickly and this is achieved through the autonomic nervous system (Tsigos & Chrousos, 2002). In a threatening situation, the sympathetic and parasympathetic nervous systems are in control of the body's reaction to this stress. The result is control over various physiological functions (Chrousos & Gold, 1992). When the stress response continues without control of the patient when there is no threat present, this can be seen as a further example of an initially adaptive condition changed into a maladaptive condition. The aforementioned condition has been named "hyperarousal" and includes symptoms reminiscent of persistent anxiety as well as autonomic hyper-responsivity (Orr & Roth, 2000).

A clear difference can be seen between fear and anxiety. Fear can be defined as an extreme, time limited emotion associated with a clearly identifiable imminent threat. Anxiety, on the other hand can be described as a generalized, continued apprehension of an imminent, but unidentified hovering threat (Marks, 1987). Moderately different neurocircuitry may be involved in these two conditions (Walker *et al*, 2003). In patients with PTSD it is found that both fear and anxiety are present; moreover, these two aggravate one another. This is yet another example of the vicious cycle at work in PTSD. In this cycle elevated anxiety and arousal cause the sufferer to feel fearful in reaction to less well-defined, unpredictable stimuli, which increases baseline anxiety with the result that the sufferer is more prone to the occurrence of a further fear condition and so the cycle repeats itself (Grillon, 2002b). As can be seen from **Table 1-1**, fear symptoms resort under the re-experiencing cluster and anxiety symptoms under the hyperarousal cluster (Bonne *et al*, 2004).

Sensitization and habituation play an important role in this particular symptom cluster of PTSD. "Habituation" can be defined as the repeated presentation of a stimulus that leads to a progressively decreasing response. A progressively increasing response is described as "sensitization". It is interesting to note that a particular stimulus can result in habituation in one instance and sensitization in another instance. Repetitive exposure to a stimulus can also lead to a more vigilant response to novel stimuli and this is called "cross sensitization". In the case of childhood trauma sufferers, enhanced sensitization and cross sensitization may be a contributing factor to the susceptibility for PTSD in these individuals (Heim & Nemeroff, 2001). Although rapid habituation of the amygdala in particular have been found in several studies, a contrasting interruption or absence in habituation is frequently reported in PTSD. Thus, it

seems that improved sensitization and delayed habituation are intricate parts of PTSD pathophysiology (Bonne *et al.*, 2004).

1.2.1 Treatment of PTSD

Everybody that endures a traumatic experience will not necessarily need treatment to recover from the psychological harm inflicted by the trauma, but several victims will need treatment to recover (APA, 2000). This presents a problem, because pharmacotherapy for PTSD is in its infancy. In America the only approved treatment currently for PTSD is selective serotonin reuptake inhibitors (SSRIs). Tricyclic antidepressants have also been used as pharmacological treatment, but did not show the same effectiveness as the SSRIs (Ballenger *et al*, 2000). The use of SSRIs seems to result in very good clinical improvement, but many patients still suffer from symptoms after treatment (Brady *et al*, 2000). It is very rarely found that a solitary drug used as treatment for PTSD can result in complete relief from the disease. Therefore a combination of drugs is mostly used and this leads to diminished patient compliance and elevated adverse events (Bonne *et al*, 2004).

There is a great lack of empirical data in pharmacological interventions in PTSD and there still exist numerous questions regarding the effective and proper use of pharmacological agents in the treatment of PTSD:

1. Are choice and efficacy of medication dependent upon PTSD phenomenology?
2. Are choice and efficacy of medication dependent upon type of trauma?
3. Should the same compounds be used in the acute and chronic conditions?
4. How long should medication be administered?

These questions remain mostly without answer (Bonne *et al*, 2004).

Table 1-2 gives pharmacological compounds that target elements of the neurobiological model for PTSD. This is not comprehensive and further research into the use of these compounds is needed.

Table 1-2: Potential therapeutic agents for PTSD (Bonne et al., 2004).

Class	**Agent**	**Mechanism of action**	**Rationale**	**Potential clinical effect**
LHPA axis components	Antalarmin	CRH-1 receptor antagonist	Impede consolidation. Decrease 5HT activation and improve learned helplessness. Reduce sensitization.	Diminish intensity of conditioned response. Diminish arousal.
	ASV-30	CRH-2 receptor antagonist.	Prevent learned helplessness.	Lessen avoidance, improve interpersonal behaviour.
	Spironolactone	MR antagonist.	Impede consolidation/ reconsolidation. Reduce sensitization.	Reduce intensity of conditioned responses. Reduce non specific arousal responses.
	Mifepristone (RU-486)	GR antagonist	Impede consolidation/ reconsolidation.	Reduce intensity of conditioned responses.
	SSR149415	AVP-1b receptor antagonism. Reduced HPA axis drive.	Impede consolidation/ reconsolidation. Oppose learned helplessness. Decrease CRH effect.	Anxiolysis; Reduce avoidance, enhance coping and improve mood.

	DHEA	Modulate GABA-A receptor; Reduce cortisol/ DHEA ratio; Excitotoxicity neuroprotection.	Attenuates contextual fear conditioning. Alleviates "learned helplessness".	Reduce environmentally incongruent conditioned fear responses. Improve mood and goal directed activity.
Catecholaminergic agents	Propranolol	β-adrenergic antagonist.	Obstruct consolidation/ reconsolidation. Inhibit recall. Reduce anxiety.	Reduce intensity of cue and context conditioned responses. Decrease non-specific arousal responses.
	Prazocin	Alpha adrenergic antagonist.	Block arousal, inhibit sensitization, obstruct learned helplessness.	Reduce anxiety, improve sleep/ nightmare effect.
Glutamatergic agents	LY35470 (or similar)	Group II 2/3 NMDA metabotropic agonist, reduces glutamatergic neurotransmission.	Disrupts fear learning and conditioned response. Neuroprotective: Reduction in NMDA neurotoxicity, increases TGF levels.	Lessen intrusive memories, reduce conditioned responses. Inhibit arousal.
	Memantine	Low-affinity NMDA channel blocker.	Delays contextual conditioning. Provides excitotoxicity neuroprotection.	Reduces arousal and contextual conditioning responses.
	Riluzole	Inhibits glutamate release. Blunts GABA-an inverse agonist.	Modulation of LHPA axis response. Excitotoxicity neuroprotection.	Reduction of LHPA drive.

	D-Cycloserine	NMDA partial agonist.	Enhance extinction. Usage together with cognitive behavioural therapy.	Reduced cue and context conditioned responses.
Anticonvulsants	Lamotrigine	Blocks voltage-gated Na^+ channels and reduces glutamate release. Suppresses GABA(A) receptor synaptic transmission.	Disrupts fear learning, fear potentiated startle. Anti kindling/ sensitization.	Improvement mostly in re-experiencing and avoidance/ withdrawal symptoms.
	Topiramate	Blocks state dependent sodium channel. Potentiates GABA anxiolysis at non-BZD site; Blocks amygdala kainate/AMPA receptors.	Disrupt associative learning. Delay sensitization/ kindling.	Reduce intrusive memories and arousal symptoms (improves sleep)
	Valproic acid	Blocks degeneration of GABA transaminase; Block voltage-gated calcium channels. Suppresses protein kinase C.	Delay sensitization/ kindling/arousal.	Reduction in intrusive memories and hyperarousal symptoms. Improved mood and activity.
	Pregabalin, gabapentin	GABA signal modulators, *via* GABA transporter 1 (GAT1).	Delay sensitization/ kindling/arousal.	Reduce autonomic hyperarousal symptoms (improve sleep).
Serotonergic	Buspirone	5HT1-A partial agonist	Prevent sequelae of inescapable	Reduced intrusions.

agents			stress	Improved mood, increase in energy.
	Mirtazapine	Adrenergic: alpha 1 agonist, alpha 2 antagonist. Serotonin: 5HT1A agonist, 5HT2A, 5HT2C, 5HT3 antagonist.	Prevent inescapable stress sequelae. Activate serotonergic system, directly and *via* NE.	Improved mood, increase in energy, lessen intrusion.
	Nefazodone	5HT2-A antagonist. Partial 5HT and noradrenaline reuptake inhibitor.	Prevent sequelae of inescapable stress, improve coping and mood, reduce anxiety.	Improvement in intrusion and hyperarousal symptoms.

Various neuroanatomical circuits, neurotransmitter systems and neuronal mechanisms are involved in PTSD, as well as central catecholamines, serotonin and extrahypothalamic CRF. These all form targets for pharmacological intervention. In the same way, it is evident that mechanisms of kindling and sensitization regulate mood and emotional memory and this opens the door to the implementation of "anti-kindling" agents as therapy for PTSD symptoms. It has become clear that PTSD is a combination of abnormalities in various neurobiological systems – a single abnormality can not account for them all and this causes a problem in finding a single pharmacological treatment (Albucher & Liberzon., 2002). That led to the investigation into the possible use of the following classes of drugs and drugs in the treatment of PTSD:

1.2.1.1. Antidepressants

1.2.1.1.1. Tricyclic antidepressants (TCAs)

1.2.1.1.2. Monoamine oxidase inhibitors (MAOIs)

1.2.1.1.3. Selective serotonin reuptake inhibitors (SSRIs)

1.2.1.1.4. Other antidepressants

1.2.1.2. Buspirone

1.2.1.3. Mood stabilizers

1.2.1.3.1. Lithium

1.2.1.3.2. Anticonvulsants

1.2.1.3.3. Benzodiazepines

1.2.1.4. Antipsychotic agents or neuroleptics

1.2.1.5. Adrenergic agents

1.2.1.6. Opioid antagonists (Albucher & Liberzon, 2002).

1.2.1.1. Antidepressants

A great deal of the initial research into the treatment of PTSD focussed on the antidepressants due to the high comorbidity between PTSD and depression as well as the common clinical features shared by PTSD and the other anxiety disorders (Albucher & Liberzon., 2002).

1.2.1.1.1. Tricyclic antidepressants (TCAs)

These drugs are blockers of the reuptake of noradrenaline and serotonin. The modulation of arousal level, stress response, mood regulation and anxiety is in part regulated by central catecholamines and serotonin (Mc Ewen, 2000). The various components of PTSD syndrome are indicative of the fact that this disorder entails dysregulation in one or more of the abovementioned functions (Kosten *et al*, 1987). Therefore, if we consider the neurobiology, it would be logical to target these systems in order to find an effective treatment for PTSD (Newport & Nemeroff, 2000).

1.2.1.1.2. Monoamine oxidase inhibitors (MAOIs)

These drugs' action is largely mediated by the same mechanisms as that of the TCAs. Resulting from this is the notion that higher levels of catecholamines and serotonin can correct abnormalities in the central nervous system caused by trauma. Currently secondary adaptive mechanisms are being explored as an explanation for the effectiveness of these drugs (Albucher & Liberzon., 2002).

When all published data, including those from open trials, are taken into account, MAOIs appear to produce moderate to good clinical improvement, mainly affecting the intrusive recollections, nightmares and PTSD flashbacks. On the contrary, hyperarousal, numbing and avoidance behaviour are not affected (Friedman, 1998).

1.2.1.1.3. Selective serotonin reuptake inhibitors (SSRIs)

Animal models and human studies have shown that increased impulsivity, aggression, fear and sadness/depression can be a result of diminished serotonin levels (Hashimoto *et al*, 1999). Furthermore, mouse knock-out models have shown that normal $5HT_{1A}$ function is necessary for the regulation of anxiety responses. Therefore, it is clear that SSRIs and SRIs enhance serotonergic function with resulting improvement in modulation of anxiety, anger, mood and impulsivity, which explain their effectiveness as treatment (Parks *et al*, 1998)

1.2.1.3. Mood stabilizers

1.2.1.3.2. Anticonvulsants

The kindling model has been identified as a possible pathophysiological abnormality underlying mood oscillations and when the anticonvulsants were found to have anti-kindling effects, it served as a possible explanation for the drugs' pharmacological effects (Weiss & Post, 1998). Kindling phenomena have been found in limbic structures such as the amygdala (Adamec & Shallow, 2000) and it is now thought that the amygdala may become kindled or sensitized after the experience of a trauma, due to inflated noradrenergic input from the locus coeruleus (Post *et* al, 1997) which leads to exaggerated fear responses, mood instability, anger and aggression. It therefore makes sense to see anticonvulsant drugs with anti-kindling properties as potential PTSD treatment. It is clear that these drugs improve intrusive symptoms and emotional lability and they reduce hypervigilance and startle response (Albucher & Liberzon, 2002). Lamotrigine, specifically, has anti-kindling properties, as seen in a study done by Otsuki *et al.* (1998) where it was found that lamotrigine potently and dose-dependently suppressed kindled seizures, with the effect lasting as long as 24 hours.

One controlled trial that has been published on the use of anticonvulsants in the treatment of PTSD was conducted as follows: in a small sample study of 15 patients, lamotrigine was compared to placebo in a double blind fashion for a 12-week period. The active medication was twice as effective, especially in the areas of re-experiencing, avoidance and numbing symptoms (Hertzberg *et al.*, 1999).

It should be noted that Wells (2003) acknowledged lamotrigine as augmentation therapy for PTSD, starting with a dose of 25 mg four times a day and a dosage range of 50 – 500 mg/day.

With the abovementioned information taken into account the current study was designed to also test the possible anxiolytic effect of lamotrigine in an animal model of PTSD. Lamotrigine will be discussed in more detail in paragraph 1.2.2.

1.2.1.3.3. Benzodiazepines

These drugs, known as anxiolytics, are active in the GABA system, where they act as potentiators of GABA-ergic neuronal circuits. This group seems to be a logical choice as treatment for anxiety, but there are many positive and negative consequences of this choice of treatment that need careful consideration before treatment is started (Albucher & Liberzon., 2002).

As has just been mentioned, this group of drugs seem to be a logical choice for the treatment of PTSD and therefore diazepam, one of the prototype drugs of this group, was chosen to use in the current study. The possible anxiolytic effect of diazepam was tested in an animal model of PTSD. Diazepam will be discussed in more detail in paragraph 1.2.2.

1.2.1.4. Antipsychotic agents or neuroleptics

Low doses of antipsychotics are used infrequently to treat anxiety. According to Weiss (1977) they are not as effective as benzodiazepines.

1.2.1.5. Adrenergic agents

It has been shown by various reports that adrenergic agents are effective in treating some PTSD symptoms, such as nightmares and hyperarousal symptoms. There are currently very promising preliminary reports of the potential use of adrenergic agents for prevention of PTSD if it is used directly post trauma (Pitman *et al*, 2002). This is very significant, because the prevention of PTSD is an important therapeutic goal. Early hyperreactivity of physiologic

measures is very much improved and this can lead to a preventive effect on the future development of PTSD (Albucher & Liberzon., 2002).

To provide a summary of the treatment decisions for PTSD, the following points should be considered:

- Current treatment of choice – an SSRI.
- Switching – a second generation anti-depressant.
- Augmentation – a mood stabilizer; an atypical antipsychotic.
- Other options – nefazodone, venlafaxine (Leonard, 2003).

Empirical evidence show that pharmacotherapy improve PTSD and symptoms in approximately 70% of patients. These improvements vary from moderate to marked improvement. A mounting body of recent evidence suggests that the antidepressants are the most effective treatment and that the SSRIs most probably have the greatest efficacy of any single class of medication (Ballenger, 1999).

The way forward for the pharmacotherapy for PTSD looks hopeful. If the pathophysiology of PTSD and its risk factors can be better understood, the outcome could ultimately be the development of more specific pharmacological agents and possibly of early intervention and prevention strategies (Albucher & Liberzon., 2002).

1.2.2. Diazepam and Lamotrigine:

These two drugs were used in the current study and will be discussed in more depth.

1.2.2.1. Diazepam

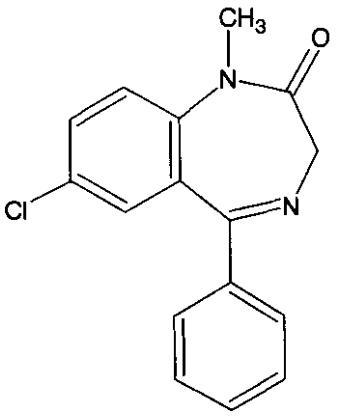


Figure 1-2: The structure of Diazepam

1.2.2.1.1. The mechanism of action of Diazepam

Benzodiazepines exert most of their effects by interacting with inhibitory neurotransmitter receptors directly activated by GABA. The ionotropic GABA-A receptors are composed of five subunits that coassemble to form an integral chloride channel. GABA–A receptors are responsible for most inhibitory neurotransmission in the central nervous system (CNS). Benzodiazepines act at the GABA-A receptors by binding directly to a specific site that is separate from that of GABA binding on the receptor/ion channel complex. Benzodiazepines do not activate GABA-A directly, but call for GABA to express their effects, meaning they only modulate the effects of GABA (Charney *et al.*, 2001: 403). At therapeutically relevant concentrations, benzodiazepines act at subsets of GABA–A receptors and increase the frequency, but not duration, of openings at GABA-activated chloride channels (McNamara, 2001: 537).

1.2.2.1.2. Pharmacokinetic parameters of diazepam:

The following values were obtained from the literature:

Humans:

- Availability: oral: 100±14 %; rectal: 90%.

- Urinary excretion: < 1%.
- Bound in plasma: 98.7±0.2%.
- Clearance: 0.38±0.06 ml.min^{-1}.kg^{-1}
- Volume of distribution: 1.1±0.3 litres/kg.
- Half-life: 43±13 hours.
- Peak time: oral: 1.3±0.2 hours; rectal: 1.5 hours.
- Peak concentrations: IV 400-500 ng/ml; oral: 317±27 ng/ml; rectal: ~ 400 ng/ml (Thummel & Shen, 2001).

Rats:

- Half-life: 1.1±0.2 hours.
- Clearance: 81.6±10.5 ml.min^{-1}.kg^{-1}.
- Volume of distribution: 3.1±0.61 litres/kg (Klotz *et al.*, 1976).

From these values it can be seen that the clearance and half-life in rats are faster than in humans.

1.2.2.2. Lamotrigine

Lamotrigine is a phenyltriazine derivative initially developed as an antifolate agent and is pharmacologically classified as an anticonvulsant (McNamara, 2001: 539).

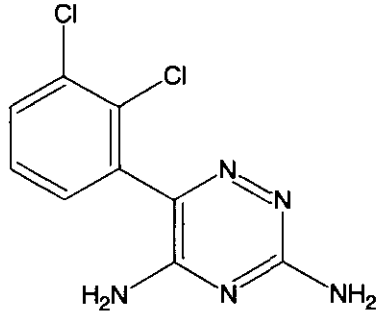


Figure 1-3: The structure of Lamotrigine

1.2.2.2.1. The mechanism of action of Lamotrigine

The mechanism underlying lamotrigine's broad spectrum of actions is not fully understood. It is known that lamotrigine blocks sustained repetitive firing of mouse spinal cord neurons and delays the recovery from inactivation of recombinant sodium channels. However, lamotrigine is effective against a broader spectrum of seizures than phenytoin and carbamazepine which suggests that lamotrigine may have a greater function than regulating recovery from inactivation of sodium channels. One explanation of lamotrigine's action could involve lamotrigine's inhibition of glutamate release, raising the possibility that lamotrigine inhibits synaptic release of glutamate by acting at sodium channels themselves (McNamara, 2001: 539).

1.2.2.2.2. Pharmacokinetic parameters of lamotrigine:

Certain pharmacokinetic parameters of lamotrigine in humans and rats were found to be as follows:

Humans:

- Availability: oral: 97.6±4.8 %.
- Urinary excretion: 10%.
- Bound in plasma: 56%.
- Clearance: 0.38-0.61 $ml.min^{-1}.kg^{-1}$
- Volume of distribution: 0.87-1.2 litres/kg.
- Half-life: 24-35 hours.
- Peak time: oral: 2.2±1.2 hours.
- Peak concentrations: 2.5±0.4 µg/ml (Thummel & Shen, 2001).

Rats:

- Half-life: 30 hours.
- Peak time: intraperitoneal injection: 0.2-0.5 hours (Walker *et al.*, 2000).

In a study by Walker *et al.* (2000) it was found that post intraperitoneal administration of lamotrigine, the drug rapidly appeared in the serum which suggests ready penetration from the peritoneal cavity. The serum pharmacokinetics are biphasic with the first phase probably representing distribution from the blood compartment and the second phase representing

mainly elimination. The prolonged elimination phase in rat is due to rat's inability to efficiently conjugate lamotrigine and this method of elimination differs significantly between rat and man (Dickins *et al.*, 1995). These kinetic effects would predict a prolonged action of lamotrigine in rat models of epilepsy and are important in the determination of lamotrigine's effects in chronic animal models (e.g. kindling) (Walker *et al.*, 2000).

To conclude a concise review of the topics that have been explored in this chapter follows. Firstly anxiety was discussed briefly and it was established that PTSD forms part of the group of anxiety disorders. The three clusters of PTSD symptoms were discussed in detail followed by a review of the pharmacological treatment options for this disorder where the drugs and classes of drugs that can be used as treatment in this disorder were discussed. Finally the drugs used in the current study were discussed.

Chapter 2: The Neurobiology and Neurochemistry of Posttraumatic stress disorder (PTSD)

2.1 Neurobiology of PTSD:

The biological basis of PTSD remains illusive, but there is evidence of a hypofunctional hypothalamic-pituitary-adrenal axis (HPA axis) and a hyperfunctional sympathetic system (Leonard, 2003). Brain areas involved in the pathophysiology of PTSD include the HPA axis, the amygdala, the hippocampus and the prefrontal cortex (PFC) (Yehuda, 2000, Sala *et al.*, 2004, Shin *et al.*, 2004). It has been proven that exposure to a traumatic experience can result in fear conditioning and this leads to the activation of the amygdala and associated structures such as the hypothalamus, locus ceruleus, periaqueductal gray and parabrachial nucleus. This activation as well as autonomic neurotransmitter and endocrine activity cause many of the symptoms of PTSD. Linked to the abovementioned activation is the orbitofrontal cortex, which produces an inhibiting effect on this activation, but in patients with PTSD the orbitofrontal cortex seems to have diminished ability to exert this inhibiting function. The hippocampus may exert a modulating effect on the amygdala (Gore, 2002) and studies have indicated a reduced hippocampal volume in PTSD patients, which may relate to the observed short-term memory deficits (Leonard, 2003).

It is important to note that the biology of PTSD is fundamentally different from the biology of stress, due to the fact that it describes a process that occurs well after the stress is no longer physically present. Therefore it can be deduced that the essential question of the biology of PTSD is one of delineating why there has been a failure of the body to return to its pretraumatic state (Yehuda, 2000).

2.1.1. *Hypothalamic – pituitary – adrenal axis (HPA axis):*

2.1.1.1. The structure of the HPA axis:

This axis consists of the hypothalamus, the pituitary and the adrenal glands. It is also one of the stress response systems of the body, along with the sympathetic-adrenal-medullary system (Shea *et al.*, 2004). The HPA axis links to the sympathetic axis *via* the locus ceruleus and the release of catecholamines such as noradrenaline follows (Strohle & Holsboer, 2003).

2.1.1.2. The role of the HPA axis in PTSD:

HPA axis dysregulation is a distinctive abnormality of PTSD that sets it apart from other psychiatric disorders (Yehuda, 2000). HPA axis activation is a key component of the human stress response and has been recognised as such since the influential work of Hans Selye, who called it the "general adaptation response" (Friedman, 2000). The HPA axis activates and coordinates the stress response by receiving and interpreting information from other areas of the brain such as the amygdala and hippocampus, as well as from the autonomic nervous system. As depicted in **Figure 2-1**, this process occurs as follows: a hormonal cascade is initiated in response to the stressor with the release of corticotrophin releasing hormone (CRH) from the hypothalamus. This leads to the release of adrenocorticotropin releasing hormone (ACTH) from the pituitary gland after which ACTH causes the breakdown of pregnanolone to cortisol in the adrenal cortex. As a result cortisol is secreted into the blood circulation. Glucocorticoids provide negative feedback at the hypothalamus, the pituitary and the hippocampus, which will shut off the stress response. Through the HPA axis, the necessary adaptive mechanisms are provided to maintain homeostasis in times of increased stress. These responses are usually protective, especially in acute situations, but they can be destructive if the hormones are overproduced or dysregulated over long periods of time (Shea *et al.*, 2004).

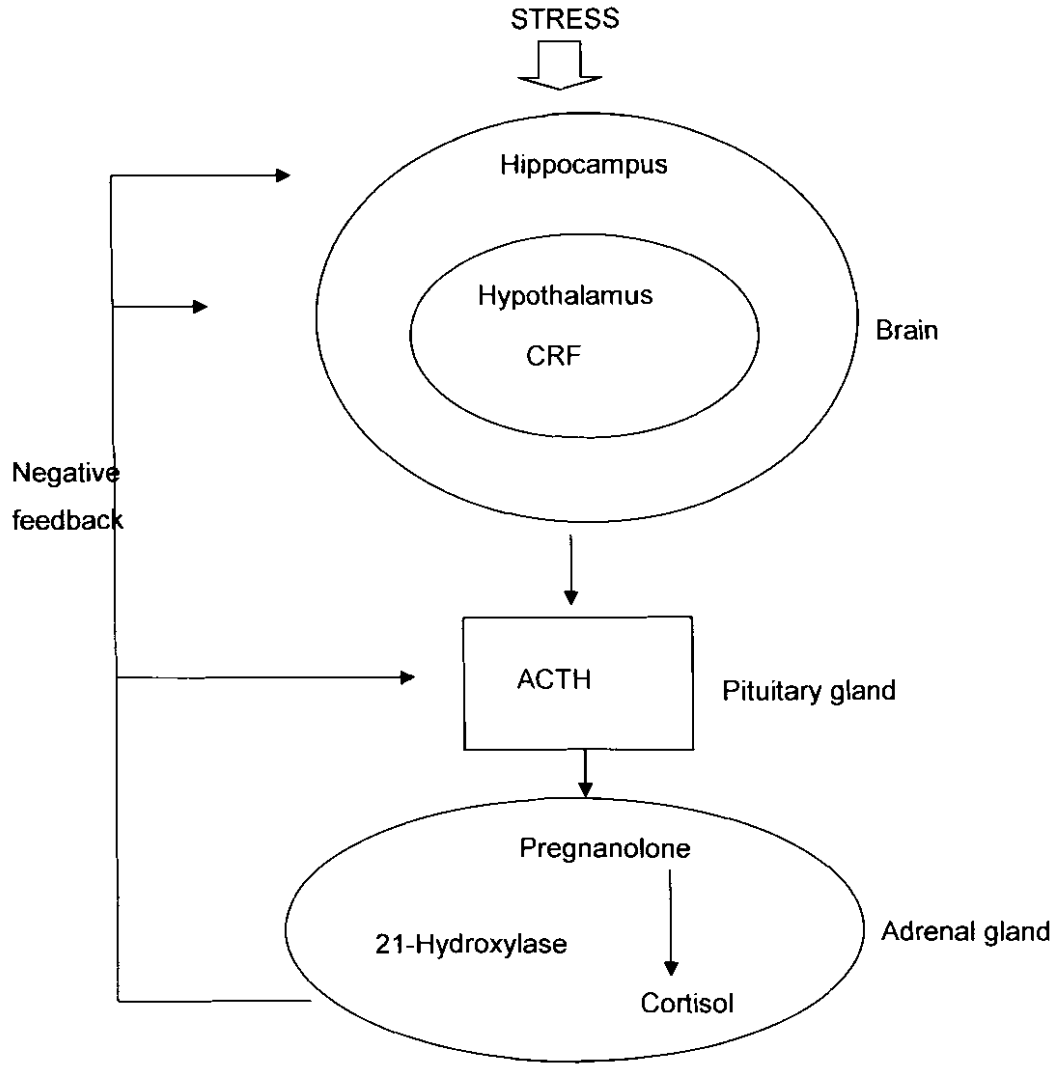


Figure 2-1: The hypothalamic-pituitary-adrenal (HPA) axis (Shea et al., 2004).

As mentioned in the previous paragraph, PTSD patients exhibit a unique HPA profile which is characterized by lower cortisol levels, upregulation of glucocorticoid receptors and supersuppression to dexamethasone (Yehuda, 2000). The enhanced negative feedback of the HPA system is a well-established finding. It has also been suggested that HPA-enhanced negative feedback may underlie the stress intolerance seen in most PTSD patients who characteristically find normal, everyday inconveniences excessively upsetting and difficult to cope with (Friedman, 1998). As is evident from the previous text, the current working understanding of HPA axis alterations in PTSD is that the HPA axis is hypersensitive to negative feedback due to a primary increase in the number and sensitivity of glucocorticoid receptors. Until recently, these alterations were thought to be characteristic of chronic PTSD; however it may be that there are fundamental differences in the way the HPA axis is normally

regulated that determine how an individual will respond to traumatic stress (Yehuda *et al.*, 1998).

It should be emphasized that clinical studies on HPA axis activity during PTSD have been inconsistent with regard to the hypo activation or hyper activation of the HPA axis. The hypothesis that the HPA axis may be hypersensitive in PTSD is consistent with the more general phenomenology of increased reactivity to both explicit and implicit trauma reminders and as well as a more generalized hypervigilance in trauma survivors with this disorder (Yehuda *et al.*, 2000b). In studies reviewed by Rasmusson *et al.* (2003), cases of ACTH up and down regulation were reported which accordingly implicates HPA axis up and down regulation. As stated in section 2.1, Leonard (2003), also found evidence of a hypofunctional HPA axis.

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

2.1.2. The amygdala:

2.1.2.1. The structure of the amygdala:

The amygdala is an almond-sized and –shaped brain structure (named after the Greek word for almond) and situated in the brain's medial temporal lobe, a few inches from either ear (Black, 2001). The amygdala is a set of subcortical nuclei needed for perceiving emotions like fear and is a component of the limbic system (University of Idaho, 2005). The subcortical nuclei can generally be divided into the central and lateral nuclei, with the central nucleus being the main processing and output station to brain regions that regulate autonomic and behavioural responses in anxiety, such as the locus ceruleus, periaqueductal gray and hypothalamus. The lateral nucleus is the main receptor/input point for cortical connections from the prefrontal cortex, cingulate gyrus, hippocampus and thalamus (Le Doux, 2003).

2.1.2.2. The role of the amygdala in PTSD:

The limbic system is pivotal in the genesis of emotions, particularly anxiety (LeDoux, 1998). The various connections in the amygdala, mentioned in the previous paragraph, provide the

amygdala with control over locomotor, neuroendocrine, autonomic and respiratory responses. Several methods of research, such as lesioning, stimulation and neuroimaging studies, have been consistent in showing the amygdala's involvement in the expression, conditioning and extinction of fear or acute anxiety (Davis, 1992). It is now thought that the amygdala not only detects and organise the response to "natural" dangers, but that it may form the centre for gathering information about novel threats, so-called classical conditioning (LaBar & LeDoux, 1996). This mechanism is similar to kindling (which will be discussed in chapter 3). It is known that the amygdala has a sensitivity to kindling in conjunction with epilepsy and it is important to point out that a "cross sensitization" of defence behaviours could be induced by amygdala stimulation with a GABA agonist (Adamec, 1993). In times of stress and anxiety elevated serotonin release has also been observed in the amygdala complex. Furthermore, it has been seen that activation of 5-HT_2 receptors in the amygdala leads to amygdaloid kindling and that this activation has a profound effect on anxiety and mood (Chen *et al.*, 2003). It may then be concluded that the amygdala represents an important potential target for pharmacological agents in the treatment of PTSD (Cahill & McGough, 1998).

The amygdala is portrayed to play a pivotal part in almost all models representing a neural circuitry in PTSD. The reason for this is that the amygdala has the ability to assess the fear-producing nature of an event and plays a decisive role in the effective establishment of memory traces linked to a potential threat, as well as the amygdala's ability to exert influence in an individual's neuroendocrine, autonomic and motor responses. The amygdala and hippocampus is seen to be involved in learning and/or memory, however, information processed within the amygdala also modulates synaptic activity and function within the hippocampal formation (Protopopescu *et al.*, 2005).

Clearly much progress has been made in the understanding of the amygdala and its involvement with fear and fear learning. Due to this, fear is the emotion that is best understood in terms of brain mechanisms. Although some points of controversy remain, these can not diminish the fundamental fact that the amygdala is the heart and soul of the fear system. New findings, pouring in constantly, are building this growing, powerful database and will hopefully lead to a neurobiological understanding not only of the normal working of the fear system, but also how it breaks down in anxiety disorders (LeDoux, 1998).

2.1.3. *The Hippocampus:*

2.1.3.1. The structure of the hippocampus:

The hippocampus, in conjunction with the adjacent amygdala, forms the central axis of the limbic system. The hippocampus is formed by two interlocking sheets of cortex. If a cross-section is studied, it has a much defined laminar structure with layers visible where rows of pyramidal cells are arranged. The connections situated in the hippocampus mostly pursue this laminar format and are as a rule uni-directional. They form well-defined closed loops that initiate primarily in the adjoining entorhinal cortex. The different cell layers and sections are defined by the series of connections made. Main pyramidal cell layers can be identified and these are the CA1-4 regions (mainly CA1 and CA3) and the dentate gyrus (University of Bristol, 2003).

2.1.3.2. The role of the hippocampus in PTSD:

As stated in paragraph 2.1.3.1, the hippocampus forms an integral part of the limbic stress pathway and this circuit is most commonly activated by psychological stressors such as restraint, fear conditioning and exposure to a new environment (Sala *et al.*, 2004). The "glucocorticoid cascade hypothesis" states that elevated glucocorticoid levels, as a result of stress, could lead to a diminished hippocampal inhibitory effect on the HPA axis, which, over time, causes hippocampal damage (Sapolsky *et al.*, 1985). This hypothesis is now supported by various preclinical studies which produced findings indicating that excessive stress or glucocorticoid exposure can damage the hippocampus, which is a major neural glucocorticoid target site with many glucocorticoid receptors (Sapolsky, 2000a). A number of structural magnetic resonance studies have reported smaller hippocampal volumes in patients with chronic PTSD (Wignall *et* al., 2004). This decline could be as a result of repeated stressful events, leading to hippocampal atrophy. A consequence of the mentioned atrophy may be the disruption of the neural circuitry underlying cognitive functions. Specifically, this may cause impairment of memory procedures, such as intrusive recollections and flashbacks. Similar mechanisms, either psychopathological or pathophysiological, such as increased levels of cortisol or glutamate, decreased neurogenesis or neurotrophic factors and glial cell loss, might contribute to shrinking hippocampal anatomy (Sala *et al.*, 2004). The frequent occurrence of high levels of glucocorticoids, as a result of stress, causes dendritic remodelling and inhibition of neurogenesis. It is clear that this could partly explain hippocampal atrophy related to stress (Sapolsky, 2000a). It should be noted that a study conducted by Brown *et al.* (2004) potentially

verifies the theory that excess exposure to glucocorticoids leads to hippocampal atrophy. They found patients exposed to chronic corticosteroid therapy to have smaller hippocampal volumes than normal controls. Furthermore, it is also important to note that Villarreal *et al.* (2002) found an association between severity of PTSD symptoms and loss of left hippocampal volume.

It should be emphasised that longitudinal, prospective MRI studies are needed in subjects with high risk of developing PTSD, to clarify whether hippocampal volume loss is a pre-existing condition (perhaps determined by a genetic vulnerability) or a result of HPA axis dysregulation (inherited or determined by traumatic experiences), or both (Sala *et al.*, 2004). Furthermore, Wignall *et al.* (2004) found that patients who have very recently developed PTSD also exhibit diminished hippocampal volumes. This result adds to the debate on whether PTSD causes the decrease in hippocampal volume or whether this pre-exists the illness. This study definitely indicates though, that hippocampal changes occur earlier in the development of PTSD than at the specific time points other studies were conducted.

It is however very important to note that the finding of reduced hippocampal volume in PTSD is not indisputable. There have been studies done where a diminished hippocampal volume in PTSD was not found and among these is a study done by Bonne *et al.* (2001). From this study it was concluded that smaller hippocampal volume is not a necessary risk factor for developing PTSD and does not occur within 6 months of expressing the disorder. It was concluded, however, that this brain abnormality might occur in individuals with chronic or complicated PTSD. The difference in the results obtained in this study from the results of those studies mentioned earlier in this section that found reduced hippocampal volumes may possibly be explained by any of the following reasons: first, structural damage to the hippocampus may result from exposure to prolonged traumatization, such as child abuse or a 1-year war experience in Vietnam. Secondly, it is possible that more than 6 months of expressing PTSD are required to produce discernable reduction of the hippocampus. Third, the previous results could have been due to substance abuse or alcohol consumption, not properly accounted for by retrospective questionnaires. Finally, a smaller hippocampus may confer a specific vulnerability to highly chronic PTSD (Bonne *et al.*, 2001).

With regard to cognitive functions, the hippocampus is involved in the formation of "declarative memory", which involves representations of facts and events that are subject to conscious recollection, verbal reflection and explicit expression (Brewin, 2001). Furthermore, the amygdala and hippocampus, along with the anterior cingulated and medial prefrontal cortex (MPFC), are involved in "conditioned fear responses". As soon as information with regard to a threat reaches the amygdala, a series of behavioural and neuroendocrine responses

immediately commence. Cortical structures and the hippocampus are also reached by the information, with these structures projecting independently to the amygdala and are essential for modulation and extinction of fear responses (Brewin, 2001). The hippocampus and the cortical structures also process more complex information, allowing creation of declarative memory of the event, in addition to the emotional memory stored by the amygdala (Sala *et al.*, 2004).

2.1.4. The frontal cortex:

2.1.4.1. The structure of the cortex:

The cortex on the orbital and medial surface of the frontal lobe is a large and heterogeneous cortical region that covers the medial wall and ventral surface of the frontal lobe.

The prefrontal cortex (PFC) is a heterogeneous region of the brain and includes the prelimbic cortex, infralimbic cortex, anterior cingulate and agranular insular cortices, as well as orbito-frontal areas (Cardinal *et al.*, 2002).

2.1.4.2. The role of the cortex in PTSD:

It seems that each of the subregions of the PFC, mentioned in section 2.1.4.1 has a unique link to emotional and motivational influences on behaviour (Le Doux, 2000). Furthermore, the PFC has to regulate working memory, attention, cognition, emotion and executive control, as well as control behavioural inhibition (Roberts *et al.*, 1998). The various specific aspects of this inhibition are mediated by different regions within the PFC (Dias *et al.*, 1997). Left dorsolateral prefrontal cortex (DLPFC) activation has been related to executive processes concerned with the monitoring and manipulation of the content held in posterior working memory stores. The significant lack of involvement of this particular region in PTSD during the updating process indicates an ineffective use in PTSD of the executive control systems attributed to the region (Clark *et al.*, 2003). The change in function of the PFC, after exposure to stress, can happen very distinctly and swiftly. This can be related to the fact that the PFC is extremely sensitive to its neurochemical environment. Although many transmitters/modulators probably contribute to the functional integrity of the PFC, research has indicated the importance of dopamine and noradrenaline to the functioning of the PFC (Arnsten, 1998).

The PFC is a crucial brain region with regard to the cognitive modulation and interpretation of anxiety-provoking experiences, as well as in the regulation of amygdalar activation (Kim & Gorman, 2005). The amygdala nuclei are kept at bay by the PFC when the PFC inhibits inappropriate emotional responses and facilitates planning and execution of effective behavioural responses to aversive or threatening events or situations (Van Praag, 2004). The findings from a recent study conducted by Shin *et al.* (2004), was consistent with the theory that there is an inverse relationship between PFC versus amygdalar activation during stressful tasks in PTSD patients. The PFC normally modulates activation of the amygdala and in the case of decreased prefrontal cortical function; amygdalar hyperreactivity might ensue in PTSD. The circuit between the prefrontal cortex and the amygdala appears to be a major locus of the ability to regulate negative affect. This was indicated by means of positron-emission tomography, when glucose metabolism was measured. Through this it was revealed that individual differences in metabolic activity in the amygdala are associated with levels of distress or dysphoria, i.e. the more activity, the greater the negative effect. In contrast, metabolic activity in the MPFC is inversely related to levels of activity in the amygdala, i.e. the greater the activity level in the MPFC the more positive the person's emotional state. A vast body of indirect evidence points very strongly to the fact that GABA, glutamate, serine, serotonin and dopamine systems are involved with the MPFC-amygdala control of emotional behaviour (Davidson, 2002).

In a very general sense, the PFC appears to be the logic centre behind the activation and modulation of fear and anxiety, as opposed to the emotional centre of its cohort, the amygdala. However, the interaction between these two brain structures is very complex and not yet fully understood (Kim & Gorman, 2005).

2.2 *Neurochemistry of PTSD:*

Within the complex neural network described above, neurochemical modulators may affect the activity within each of these brain areas and at nodes along the entire neurocircuitry system. It is important to note that this creates a large number of potential pharmacological targets (Kent, *et al.*, 2002). In this regard the focus will be mainly on the different neurotransmitters involved in PTSD with special reference to GABA and secondly the role of cortisol will be discussed.

2.2.1. Neurotransmitters involved in PTSD:

2.2.1.1 Noradrenaline (NA)
2.2.1.2 Dopamine (DA)
2.2.1.3. Serotonin (5-HT)
2.2.1.4. Glutamate
2.2.1.5. Gamma amino butyric acid (GABA).

In this study the emphasis will be on glutamate and GABA and these two neurotransmitters will be discussed in more depth.

2.2.1.1. Noradrenaline (NA):

2.2.1.1.1. The role of NA in PTSD:

Central noradrenergic neurons serve as elements within a diffuse modulatory system that detects and responds to meaningful internal and external stimuli. The cell bodies of most noradrenergic neurons in the brain are located within a discrete group of hindbrain nuclei, the most prominent of which is the locus ceruleus. It has been suggested that these noradrenergic nuclei are critical in determining the organism's overall state of arousal and attention (Southwick *et al.*, 1999).

The locus ceruleus processes relevant sensory information through its diverse afferent inputs and has an efferent network that can potentially facilitate anxiety and fear-related skeletal motor, cardiovascular, neuroendocrine and cognitive responses. Electrical and pharmacologic stimulation of the locus ceruleus elicits fear-related behaviours (in primates) and increased release of NA in multiple brain regions, such as the amygdala, hippocampus and prefrontal cortex. These brain regions are involved in perceiving, evaluating, remembering and responding to potentially threatening stimuli. Bilateral lesions of the locus ceruleus dramatically reduce fear behaviours and NA release in threatening situations (Charney *et al.*, 1995).

As mentioned in the previous paragraph, a consequence of stressful events or situations is enhanced NA-ergic activity, primarily in the locus ceruleus. Projections from there innervate three structures essential for learning and memory: the PFC, amygdala and hippocampus (Blier, 2001). Increased NA-ergic activation in the amygdala translates into enhanced

consolidation and facilitated retrieval of emotional events (Cahill, 1997). It is also interesting to note that findings from a study conducted by Shalev *et al.* (1998) suggest that those individuals with an excessive adrenergic response, such as elevated pulse rate, were more likely to develop PTSD than others.

A growing body of studies have provided compelling evidence for increased noradrenergic activity in traumatized humans with PTSD (Friedman & Southwick, 1995). This increased activity generally is not observed under baseline or resting conditions, but rather in response to a variety of stressors. It has been suggested that altered reactivity of NA-ergic neurons is associated with a variety of hyperarousal and re-experiencing symptoms characteristic of PTSD (Southwick *et al.*, 1997). Clinical data therefore justify the conclusion that in PTSD, NA activity is increased. In other words, fear-related neuronal circuits are in a sustained state of hyperactivity even in the absence of a specific stressor (Van Praag, 2004).

Increased responsivity of noradrenergic systems is consistent with a sensitization model of PTSD, where biochemical, physiological and behavioural responses to subsequent stressors increase over time. It has been suggested that sensitization of NA-ergic systems contributes to arousal symptoms in PTSD, including hypervigilance, exaggerated startle, anger and insomnia (Southwick *et al.*, 1999). Increased tone of the NA-ergic system and increased responsivity of that system towards both neutral and emotional stimuli might be associated with the enhanced consolidation of emotion-laden aversive events and in facilitating their retrieval, disturbances in declarative memory so typical for the PTSD patient. Repeated re-experiences of trauma-related material might cause sensitization, implying that the physiological and behavioural responses to trauma-related stressors and eventually also to innocent stimuli intensify over time rather than abate. Re-experiencing traumatic occurrences will enhance NA release, amygdala activity and emotional reactivity, setting in motion a vicious circle. Therefore it can be concluded that hyper-consolidation and facilitated retrieval of trauma-related material, sensitization for trauma-related stimuli, enhanced fear conditioning leading to fearfulness and hyperarousal, phenomena typical for PTSD, might be related to sustained NA-ergic hyperactivity (Van Praag, 2004).

Finally, it should be noted that NA released by emotional arousal enhance declarative memory which could explain why emotional events are better remembered than emotionally neutral events (Patten, 1999).

2.2.1.2. Dopamine (DA):

2.2.1.2.1. The role of DA in PTSD:

Animal studies also support the role of the dopaminergic system in the pathophysiology of acute and chronic stress (Bremner *et al.*, 1999). In rats, mild and brief stress, as well as chronic stress, results in an increase in dopamine release and metabolism in the medial prefrontal cortex (Finlay *et al.*, 1995).

It has also become evident that elevated dopaminergic activity seems to be involved with states of acute symptomatology for PTSD. Several studies suggest that this hyperactive dopaminergic system in PTSD could play a part in the pathophysiology of certain PTSD symptoms, such as emotional numbing and hypervigilance (Bremner *et al.*, 1999). Studies have also shown that PTSD patients excrete notably more urinary dopamine and its metabolite homovanillic acid with these elevated levels correlating with the severity of PTSD symptoms, most directly with intrusive symptoms. A hypothesis exist which states that DA and glutamate hyperactivity is involved in the neurobiology of PTSD (Newport & Nemeroff, 2000). A study done by Comings *et al.* (1996) provide even more evidence for dopaminergic involvement in PTSD. They found a heightened risk of developing PTSD associated with the D_2A1 dopamine receptor allele. The same study also suggest that a dopamine D_2 receptor gene variant in linkage disequilibrium with the D_2A1 allele confers an increased risk to PTSD and the absence of the variant confers a relative resistance to PTSD.

2.2.1.3. Serotonin (5-HT):

2.2.1.3.1. The role of 5-HT in PTSD:

Regulation of 5-HT receptor density and/or sensitivity plays an important role in modulating neurotransmission in the central nervous system, while changes in receptor density are seen during the development and pharmacotherapy of several anxiety and affective disorders (Siever *et al*, 1991). $5\text{-}HT_{1A}$ receptors are densely located in limbic areas, especially the hippocampus and play a key role in mediating the stress response possibly by influencing HPA axis activity (Richer *et al.*, 2002; Lopez *et al.*, 1999; Cassano & D'Mello, 2001). In particular, anxiogenic states have been associated with elevated serotonin release in the amygdala (Kirby *et al.*,

1995), whereas 5-HT depletion in the amygdala resulted in a specific anxiolytic effect (Sommer *et al.*, 2001). Thus, it is clear that pre- and postsynaptic 5-HT_{1A} receptors exert profound effects on anxiety (McKittrick *et al.*, 1995), while they perform a further, central role in spatial memory and cognition as well as mediation of the effects of stress on these functions (Gerlai *et al.*, 2002). 5-HT_2 receptors, on the other hand, preferentially populate the cortical areas, while activation of these receptors is known to be associated with severe anxiety and to impede adaptation to stressors (McKittrick *et al.*, 1995). Moreover, 5-HT_{2A} receptor activation further activates the HPA axis (Heimrick-Leucke & Evans, 2002). Recently, Kanlynchuk *et al.* (2001), have described the elevation of 5-HT_{1A} and 5-HT_{2A} receptor density in the hippocampus and PFC, respectively, in fear-sensitized rats. These changes were significantly correlated with increases in emotional and fearful behaviour, providing support for a dual role for these two receptors in the behavioural and adaptive response to stress (Harvey *et al.*, 2003).

Animal research further suggests that 5-HT affects many fundamental brain mechanisms that are altered in PTSD, including sleep regulation, aggression, cardiovascular activity, motor function, anxiety, mood and neuroendocrine secretion. Most notably, 5-HT neurons have direct effects on both adrenergic and HPA function (Friedman, 2000). Results form a study conducted by Southwick *et al.* (1997b), suggest that some PTSD syndromes may result primarily from 5-HT dysregulation, whereas others may be due to abnormal adrenergic mechanisms. The implications of this possibility for pharmacotherapy are enormous, since antiadrenergic agents might be the treatment of choice for one PTSD subtype whereas drugs that specifically target 5-HT might be needed for the other subtype.

2.2.1.4. Glutamate:

2.2.1.4.1. The synthesis of glutamate:

Glutamate, a common amino acid is found in abundance in nature. It is a natural component in virtually all protein-containing foods and many vegetables. The intestine utilizes almost all of the glutamate taken into the body by means of these foods, so much so, that the rest of the body has to synthesize nearly all of the glutamate that is needed. This is especially true for the brain where glutamate is used as a neurotransmitter. The blood brain barrier does not allow the passage of glutamate; therefore the brain has to produce its own glutamate from glucose and other amino acids (International glutamate information service, 2005).

As the major excitatory amino acid in the CNS, this single neurotransmitter is integral to the functioning of up to 40% of all brain synapses. Glutamate is primarily derived from intermediary glucose metabolism and can be formed directly from glial cell-synthesized glutamine stores. Several glutamate transporters serve to regulate synaptic transmission and synaptic cleft levels of glutamate, with astroglial transporters providing the primary means for synaptic inactivation of glutamate in the forebrain (Kent *et al.*, 2002).

2.2.1.4.2. The role of glutamate in PTSD:

Glutamate is the brain's primary excitatory neurotransmitter. It subserves nearly all fast excitatory point-to-point synaptic transmission in the brain and is rapidly released in response to arousing and dangerous situations. During resting nonstressful states, excitatory glutamatergic synaptic transmission is regulated by GABA. Tonic inhibition by GABA, in multiple brain regions such as the thalamus and amygdala, allows the brain to filter out a continuous flow of irrelevant and extraneous sensory information; however, during stressful or dangerous situations when excitation is increased, elevated levels of glutamate are able to overcome tonic inhibition by GABA and thereby trigger a cascade of protective responses (Krystal *et al.*, 1995).

Although stress-induced elevations of glutamate serve to mediate cortical and subcortical communications and responses to stress, failure to regulate and modulate heightened glutamatergic activation can lead to extreme changes in intracellular calcium, toxicity and even cell death (Thomas, 1995). To protect the brain from its own excitation, additional GABA is released during stress. Thus, in addition to providing tonic inhibition to multiple brain regions during nonstressful states, increased GABA-ergic inhibition during stress helps to contain and terminate excitation, which if left unchecked could be disorganizing and toxic (Morgan *et al.*, 2003).

Multiple cortical and subcortical regions (including sensory, motor, prefrontal and cingulate cortex, hippocampus, amygdala, thalamus, striatum, midbrain and brain stem monoaminergic nuclei and hypothalamus) become activated during stress. Communication between these regions facilitates evaluation of psychomotor response to and memory for stress-related events and is highly dependent on glutamatergic signalling. For example, glutamate is critically involved in conveyance of sensory information from periphery to thalamus; from thalamus to amygdala, hipocampus and cortex; and from amygdala, hippocampus and cortex back to thalamus (Krystal *et al.*, 1995).

PTSD is characterized by a loss of cognitive abilities with evidence of increased glutamatergic activity (Chambers *et al*, 1999). In support of this, a recent pilot study by Heresco-Levy and colleagues (2002) reports on the clinical evidence for efficacy of D-cycloserine, a partial agonist at the glycine regulatory site on the *N*-methyl-D-aspartate (NMDA) receptor, in the treatment of PTSD. Stress and glucocorticoids not only increase glutamate concentrations in the hippocampus, but glucocorticoids also selectively increase glutamate accumulation in response to excitotoxic insults in this brain region (Sapolsky, 2000b). Thus, hippocampal damage resulting from the effects of increased levels of glucocorticoids due to trauma exposure will further elevate levels of glutamate, thus potentiating the neurotoxic process. It is therefore clear that, while increased levels of glucocorticoids may initiate hippocampal damage, it also activates other neurotoxic pathways that may drive neuronal damage over a protracted period after the traumatic event (Oosthuizen *et al*., 2005).

Focussing more on the long-term sequelae of acute severe stress, it was found that time dependent sensitization (TDS) stress (discussed in sections 3.4.2 and 4.5) evokes a profound effect on glutamate receptors in the hippocampus of rats three weeks after stress exposure (Harvey *et al*, 2004). In this study TDS stress induced a significant down-regulation of hippocampal NMDA receptors. Despite the indication that overstimulation of the NMDA receptor might explain the neurodegeneration observed in PTSD, preclinical studies have found that inhibition of glutamate reuptake, resulting in increased glutamate levels, leads to a decrease in NMDA receptor density (Cebers *et al*, 1999). This is suggested as a possible neuroprotective mechanism to counteract NMDA receptor overstimulation (Naskar & Dreyer, 2001).

Among trauma victims at risk for PTSD, the factual memory registration and the biological components of emotional response could be impaired. 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 may overstimulate the NMDA system (Nutt, 2000). The overstimulation of the NMDA receptors would lead to excessive influx of calcium ions into the postsynaptic neurons. These ions are extremely toxic to cells and eventually induce cytotoxic cell death, which may be another of the key mechanisms by which brain cells could be lost in chronic PTSD (Vaiva *et al*., 2003).

2.2.1.5. Gamma amino butyric acid (GABA):

2.2.1.5.1. The synthesis of GABA:

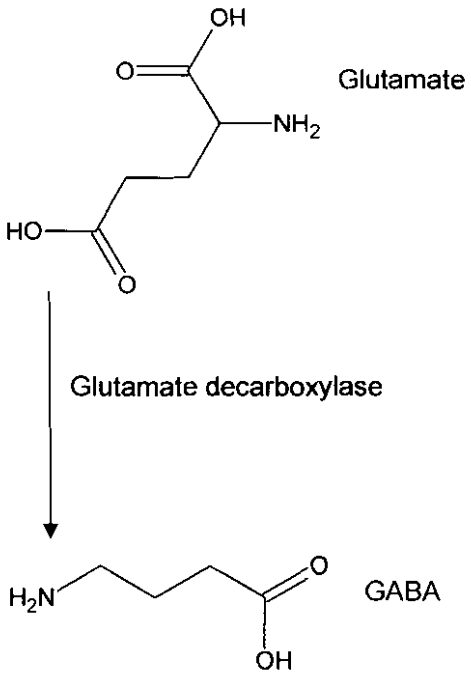


Figure 2-2: The synthesis of GABA

As depicted in **Figure 2-2**, GABA is mainly synthesized from glutamic acid by means of the enzyme glutamate decarboxylase (GAD). Molecular cloning studies have shown that in the adult brain GAD exists as two major isoforms, called GAD65 and GAD67, so named because of their approximate molecular weights of 65 400 Da and 66 600 Da, respectively. These two isoforms are the products of two independently regulated genes located on chromosomes 2 and 10, respectively, in humans (Erlander *et al.*, 1991). However, studies have shown that GAD65 is by far the most abundant form in the dentate gyrus and CA1 region of the rat hippocampus (Sloviter *et al.*, 1996). The existence of two GAD isoforms makes GABA different from other neurotransmitters, such as acetylcholine, the catecholamines or 5-HT, as each of their key synthetic enzymes is the product of a single gene (Soghomonian & Martin, 1998).

Two further reaction sequences also convert putrescine to GABA, either by deamination by diamine oxidase or *via* *N*-acetylated intermediates. The relative importance of these three

routes of GABA biosynthesis varies among tissues and with developmental stage (Rodwell, 1996).

2.2.1.5.2. The catabolism of GABA:

Transamination of GABA, catalyzed by GABA-transaminase, forms succinate semialdehyde. Succinate semialdehyde may then undergo reduction to γ-hydroxybutyrate, a reaction catalyzed by L-lactate dehydrogenase, or oxidation to succinate and thence *via* the citric acid cycle to CO_2 and H_2O (Rodwell, 1996).

2.2.1.5.3. GABA and the GABA receptor complex:

As an amino acid derivative, GABA is the most important and abundant inhibitory neurotransmitter in the brain. Acting as a "balancer", it helps induce relaxation and sleep by balancing excitation of the brain with inhibition. GABA exerts its effects by binding to two distinct receptors, GABA-A and GABA-B. The GABA-A receptors are pentameric membrane proteins that operate as GABA-gated Cl^- channels responsible for synaptic inhibition. The anxiolytic drugs of the benzodiazepine family exert their soothing effects by potentiating the responses of GABA-A receptors to GABA binding. The GABA-B receptors are coupled to an intracellular G-protein and act by increasing conductance of an associated K^+ channel (Chou, 2004). GABA receptors are found throughout the brain, including the regions implicated in anxiety disorders like the amygdala. It is possible that the diffuseness of GABA receptors in both emotional and physiological regions of the fear circuit may be responsible for the rapid calming effects that occur when they are activated (Kim & Gorman, 2005).

GABA-A receptors exhibit varied structural heterogeneity and are assembled from a repertoire of at least 18 subunits. These receptors are most clearly distinguished by their subunit architecture, which in mammalian brain comprises seven different classes of subunits with multiple variants. Most GABA-A receptor subtypes *in vivo* are believed to be composed of α, β and γ subunits. Of the 12 constituent subunits (α_{1-6}, β_{1-3} and γ_{1-3}), β_2 is ubiquitous and the most abundant subunit in the brain. The subunit γ_2 is a necessary subunit for benzodiazepine binding. For the six α isoforms, only α_1, α_2, α_3 and α_5 determine benzodiazepine pharmacology. This is because the residue His-129 of α_1 is critical for benzodiazepine binding (Chou, 2004). The equivalent position at α_4 and α_6 is occupied by Arg and its mutation to His is required for their interaction with classical benzodiazepines. Accordingly, despite numerous possible αβγ combinations, $\alpha_k\beta_2\gamma_2$ (k=1,2,3 and 5) combinations, or subtypes 1,2,3 and 5 appear to be

prototypic for GABA-A receptors, sharing many properties with those of native neuronal receptors. Of the four receptors, subtype 1 is thought to be the most abundant in the adult brain (McKernan & Whiting, 1996). On the other hand, it has been shown by mounting evidence that subtype 2 is critical for mediating the anxiolysis produced by classical benzodiazepines (Chou, 2004).

GABA-A receptor channels open after binding GABA to give a net inward flux of negative chloride ions (outward current), thus hyperpolarizing the membrane and reducing neuronal firing (Macdonald & Olsen, 1994).

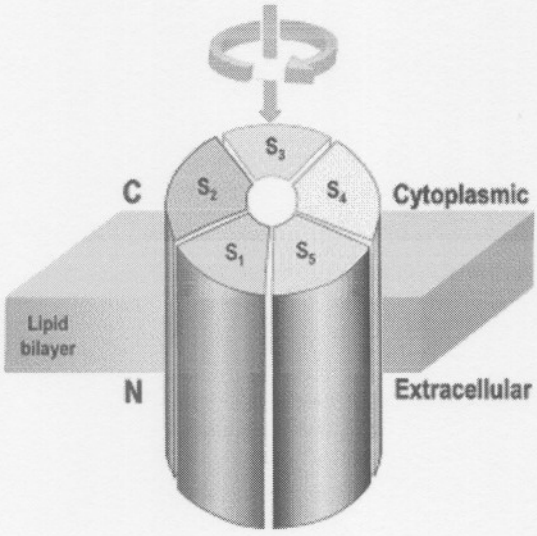


Figure 2-3: Schematic drawing to show the stoichiometry and the arrangement of the five subunits in a GABA-A receptor, with a view from C- to N-terminal (Chou, 2004).

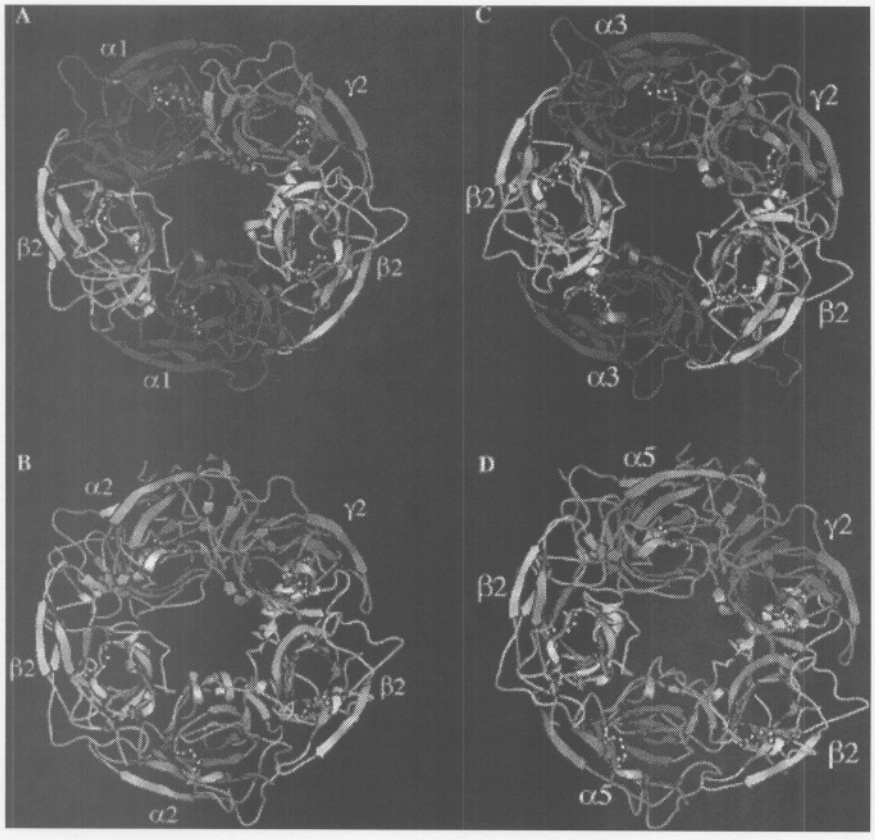


Figure 2-4: The computed structure of GABA-A receptor (viewed from C- to N-terminal) for (A) subtype 1, (B) subtype 2, (C) subtype 3, and (D) subtype 5 (Chou, 2004).

2.2.1.5.4. The role of GABA in PTSD:

GABA is known to modulate the HPA axis and the role played by this axis in PTSD is well established as described in section 2.1.1.1. GABA-ergic systems have also been implicated in the pathogenesis of anxiety, depression and insomnia, which are among the disturbances seen in PTSD (Vaiva *et al.*, 2003). These two converging lines of evidence led Vaiva *et al.* (2003) to hypothesize that the GABA-ergic system may be implicated in the modulation of PTSD

Few human studies have examined the links between GABA and PTSD (Vaiva *et al*, 2003). GABA and glutamate are involved in the process of factual memory registration and in encoding emotional and fear memory (Izumi *et al.*, 1999). Studies by Meis and Pape (2001) show that substances that increase GABA-ergic activity in the amygdala may reduce vulnerability to stress.

The natural development of PTSD is dependent on various comorbid factors, including the nature of the prior trauma and intra-subject variation. Of course a question relating to the natural development of PTSD post stress is why various people exposed to the same stressor respond differently, some developing PTSD and others not (McNally, 1998).

Harvey *et al.* (2004) made an important finding in animal studies. They found that TDS stress-induced activation of hippocampal inducible nitric oxide synthase (iNOS) and the down regulation of NMDA receptors are associated with a significant attenuation of total hippocampal GABA. The inhibition of glutamatergic transmission is mainly a function of GABA. GABA exerts this function through activation of presynaptic GABA-B heteroreceptors (Yamada *et al.*, 1999). Moreover, associated studies have found swim stress-induced GABA release in the hippocampus to be potentiated by nitric oxide (NO) (Engelman *et al.*, 2002). This is indicative of an important protective mechanism to curb excessive glutamate-nitric oxide synthase (NOS) activation through actions on GABA release. Engelman *et al.* (2002) also found acute stress to increase GABA levels which could possibly be a direct response to oppose the excitotoxic burst of glutamate. On the other hand repeated trauma, of which TDS is an example, seems to extinguish this protective effect of GABA. Within this same context, it is pertinent to note that NO also inhibits NMDA receptor-mediated GABA release (Moller *et al.*, 1995), such that raised NO depletes neuronal GABA thus leaving the excitotoxic effects of glutamate unopposed (Oosthuizen *et al.*, 2005). It could therefore be assumed that in response to chronic, persistent stress, GABA's function changes or become depleted, resulting in sub-optimal GABA function. A further assumption that can be made is that if PTSD is not treated the result would be an

increasing GABA-glutamate imbalance leading to profound neurodegenerative consequences and PTSD gradually worsening (Oosthuizen *et al.*, 2005).

It is clear that GABA plays an important part in PTSD and it would therefore be advantageous to know how GABA concentrations are affected by stress that could lead to PTSD. Acute stressors have been reported by some researchers to increase (Harvey *et al.*, 2002) and by others to decrease (Briones-Aranda *et al.*, 2005) brain GABA levels. The studies mentioned are merely two examples of the many studies that determined GABA levels in response to stress and the results from these were definitely inconclusive with regard to an increase or decrease in GABA levels. These apparent discrepancies seem to be due mainly to the stressful factor used and the time at which the biochemical determination was made (Briones-Aranda *et al.*, 2005). Furthermore, a recent paper has indicated that low post-trauma GABA plasma levels may represent a predictive factor in the development of posttraumatic stress disorder (Vaiva *et al.*, 2003). In fact, people with panic disorder also appear to present with abnormally low GABA levels (Goddard *et al*, 2004). The latter clinical data are reminiscent of that obtained in animals subjected to TDS (Harvey *et al*, 2004). All that can be concluded at this stage is that the elevation or fall of GABA levels remains a highly contentious issue.

2.2.2. *Cortisol and its role in PTSD:*

Corticosteroids are secreted by the adrenal cortex. They enter the brain and exert their effects *via* two types of receptors: the mineralocorticoid (MR) receptor and the glucocorticoid (GR) receptor. Both are found abundantly in the hippocampus. Other brain regions contain predominantly GRs (De Kloet *et al.*, 1998).

Corticotrophin releasing hormone (CRH) and other neuropeptides are expressed by the neurons in the paraventricular nucleus (PVN) of the hypothalamus. The activity of the HPA axis and the sympatho-adrenomedullary system are generated by these peptides. Both these systems exert control over each other's function (De Kloet *et al.*, 1998). It is the HPA axis that secrete corticosterone in the rat (Wong *et al.*, 1994) and cortisol in the human (Retana-Marquez *et al.*, 2003).

Corticosteroids enable an individual to cope with stress, i.e. to withstand disturbances in homeostasis and correct them once they have occurred. In part, this function is fulfilled by controlling the excitability of neuronal networks underlying learning and memory (De Kloet *et al.*, 1999).

Baseline studies in adult PTSD patients report high levels of CRH in cerebrospinal fluid (CSF) (Baker *et al.*, 1999), while diurnal plasma cortisol levels on average are decreased (Yehuda *et al.*, 1994). Single point plasma and 24h urine cortisol measurements, however, reveal mixed results. Lower plasma and 24h urine cortisol levels have been reported in some (Yehuda *et al.*, 1995a, b) but not in other studies (Lemieux & Coe, 1995). Twenty-four hour urinary cortisol studies in PTSD that have more carefully controlled for factors of relevance to HPA axis activity tend to show higher cortisol output among PTSD patients compared with controls. This is not to say that high cortisol output characterizes PTSD. There still is not enough conclusive data to distinguish among the following possible explanations for the variability among these studies. Cortisol hyperreactivity is characteristic of HPA axis function in PTSD and this phenomenon is observed readily in outpatients, but not when subjects are confined to nonprovocative environments. In addition, lower cortisol output is characteristic of more severely ill PTSD patients with increased avoidance symptoms, while less ill PTSD subjects are recruited to outpatient studies and show higher cortisol output. Finally, factors related to gender or premenopausal state within female gender contribute to increased cortisol output in PTSD. Further research will be required to clarify these issues (Rasmusson *et al.*, 2003).

The combination of high CRH in CSF and lower baseline or diurnal plasma cortisol levels have often been attributed to the enhanced HPA axis feedback regulation in PTSD, described in section 2.1.1.1. Other mechanisms that might be involved are an altered perception of stressors, alterations in activity of the central nervous system, alterations in hormone bioavailability and hormone receptor function (De Kloet *et al.*, 2005). In the same study De Kloet *et al.* (2005) reviewed 26 challenge studies, using different methods to assess HPA axis functioning. Key findings of the reviewed studies are enhanced salivary cortisol levels in response to cognitive challenge, as well as enhanced plasma cortisol suppression after administration of 0.5 mg of dexamethasone.

It is also important to point out that 24 hour urinary free cortisol levels may be influenced by more prolonged or habitual patterns of activity. One study found low cortisol output in PTSD (Yehuda *et al.*, 1995a): cortisol output correlated negatively with avoidance symptoms that, in turn, were very high among the subjects with PTSD.

The observation of low cortisol levels in PTSD was initially considered counterintuitive, because cortisol levels have generally been found to be high in conditions of acute and chronic stress and in certain types of psychiatric disorders that are associated with stress. In cases of chronic stress or chronic illness such as depression, increased cortisol levels usually indicate that the HPA axis has grown resistant to the effects of cortisol. This cortisol resistance can be

measured by the extent of "nonsuppression" of cortisol after administration of dexamethasone. Indeed, about half of patients with major depressive disorder demonstrate a nonsuppression of cortisol on the dexamethasone suppression test. In contrast, low cortisol levels in PTSD are associated with enhanced cortisol suppression after dexamethasone administration, suggesting that the HPA axis may actually be overly responsive to stimulation (Rasmusson *et al.*, 2003).

As stated previously in this section, relative to the amount of stress, cortisol levels in plasma and urine are low in PTSD and cortisol suppression after low doses of dexamethasone is above normal (Southwick *et al.*, 1998). It could therefore be deduced that corticosteroid receptors are understimulated, particularly GRs that have 10 times lower affinity for cortisol than MRs. PTSD patients show amnesia signs of diminished consolidation and/or retrieval of newly learned material and inability to eliminate dysfunctional reaction patterns. Memory deficits are not only present in chronic PTDS patients but are demonstrable within 10 days of the trauma. Low cortisol response has likewise been ascertained in the immediate proximity of the trauma (Southwick *et al.*, 1998). These cognitive features may be related to GR understimulation. No PTSD symptoms suggest MR understimulation (Van Praag, 2004).

As described in paragraph 2.1.3.2, glucocorticoids negatively influence the hippocampus in reaction to stress. It is now thought that this effect of glucocorticoids could possibly explain, in part, the acutely reversible as well as chronic effects that stress has on declarative memory (De Kloet *et al.*, 1999).

Stress can significantly modulate memory consolidation (McGaugh and Roozendaal, 2002) and retrieval (de Quervain *et al.*, 1998). Memories susceptible to modulation by stress include conditioned fear-related memories, including memories of both contextual stimuli (Conrad *et al.*, 1999) and specific cues (Conrad *et al.*, 1999). Considerable evidence has also demonstrated that corticosterone activated GRs can directly modulate memory in both animal models (McGaugh and Roozendaal, 2002) and in humans (de Quervain *et al.*, 2000). Oral cortisol impairs the retrieval of negative but not neutral words in human subjects (Khulmann *et al.*, 2005). In agreement with stress models, manipulation of endogenous levels of corticosterone either by direct injection or oral administration with corticosterone, or with GR pharmacological manipulation, results in an enhancement and/or inhibition of several forms of fear-related learning. These include passive avoidance learning (McGaugh, 2000) and Pavlovian conditioning to discrete stimuli (Hui *et al.*, 2004) and context (Conrad *et al.*, 2004). In addition, Pavlovian conditioning is also modulated by GR agonists (Zorawski & Killcross, 2003). The role of glucocorticoids in animal models will be discussed in more detail in chapter 3.

As is evident from this section, studies in a variety of populations have yielded varied results, suggesting that there may not be a characteristic pattern of cortisol output associated with PTSD (Rasmusson *et al.*, 2003).

At this point, it may be asked whether a uniform pattern of findings in complex neuroendocrinologic systems regulated at so many points and subject to controllable as well as uncontrollable environmental influences should be expected to be associated with the heterogeneous symptom complex we now call PTSD. It should already be clear that the answer to this question is no. This is not surprising. As illustrated by numerous animal studies, steroids, such as cortisol, impact the neural substrate at many levels and interact with numerous neurotransmitters and other factors that react to environmental provocations. The resulting alterations in brain state – both immediate and long term – must then be the product of a medley of inputs acting on variably vulnerable neural substrates. To put it simply, we might expect that for each person with PTSD who exhibits relatively high or low cortisol output, there is a healthy individual with the same cortisol output, differing with respect to other factors that render that individual more stress resilient (Rasmusson *et al.*, 2003).

Chapter 3: Animal Models of Posttraumatic Stress Disorder and Anxiety

3.1 Introduction:

Animal models have become extremely useful tools in the investigation of psychiatric disorders. The value these models add to scientific investigation are illustrated by the following:

- These models can determine how behavioural symptoms and biological abnormalities are linked (Yehuda & Antelman, 1993).
- These models are utilised to test drugs as possible treatment for psychiatric illnesses (Yehuda & Antelman, 1993).
- The scientific investigator has the option of simulating a human condition under controlled circumstances when using these models (Yehuda & Antelman, 1993).
- ❖ In these models a disorder can be studied as it develops which also mean the disorder's symptoms can be studied during development. This is in contrast to human disorders, which can be studied only after they become clinically manifest (Yehuda & Antelman, 1993).

It should be noted that specifically PTSD is a disorder that lends itself to be easily modelled, because the major precipitating factors, anxiety and stress (response to severe traumatic situation) are known (Yehuda & Antelman, 1993).

PTSD is classified as an anxiety disorder (APA, 1994) with specific symptoms precipitating after exposure to a traumatic event (APA, 1994). Therefore, the rest of this chapter will focus on methods of inducing anxiety, animal models of anxiety and animal models of PTSD.

3.2 Methods of inducing anxiety:

3.2.1. Pavlovian conditioning:

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

This neural mechanism involves the amygdala, locus ceruleus, hippocampus and thalamus (Rosenweig *et al.*, 1996). Moreover, there have been general clinical observations in PTSD patients that elicit symptoms such as anxiety and hyper-arousal ultimately leading to the frequent re-experiencing of the trauma, all of which could be attributed to fear conditioning (Cardinal *et al.*, 2002). This notion is supported by Maren (2001) whom have suggested the mechanism of fear conditioning as a model for the re-experiencing symptoms of PTSD due to the association between traumatic recall and seemingly unrelated stimuli and the end result of fear.

Exposure to a severe traumatic event would lead to fear conditioning, which is adaptive if the fear conditioning continues only for the duration of the threat. However, occasionally the fear response may persist even after removal of the threat and/or removal from the threatening context. It is when this situation occurs that stimuli that is only vaguely related to the trauma suffered, may lead to the fear response. This then reflects sensitisation and cross sensitisation of the neuronal system with a subsequent observation of all the symptoms of PTSD (re-experiencing; avoidance; hyperarousal).

Referring back to chapter 1, figure 1-1 represents a cascade of events strengthened by enhancement of fear conditioning and by delayed escape behaviour that are both part of the learned helplessness syndrome. In turn, there exist mechanisms that may enhance maladaptive fear conditioning and these are: enhanced recall, deficit in integration of context and content and impaired alternative learning (Bonne *et al.*, 2004).

Conditioned fear responses have mostly been studied to gain knowledge about the mechanisms underlying particular cognitive-affective processes and the subject of these studies has most frequently been rodents (Bonne *et al.*, 2004). 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 a 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 resonse to previously neutral stimuli (Le Doux, 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).

3.2.2. Kindling / sensitisation:

In PTSD there appear to be both sensitisation and kindling phenomena (Post *et al.*, 1997). A component of stressor sensitisation is often clinically apparent, as individuals who have experienced prior traumatic life events appear more likely to suffer from PTSD upon repeated exposure to trauma (Yehuda, 1995c). Repeated inducing events may also yield more severe and long-lasting consequences than isolated events, even of severe or horrific magnitude. Moreover, there may be a kindling progression in the emergence of flashbacks, which initially may be triggered by cues linked to the original event and then begin to occur more spontaneously (Post & Weiss, 1998)

Kindling is traditionally defined as an increase in seizure activity when a subthreshold stimulus is applied repeatedly to certain brain structures. In the kindling model repeated electrical stimulation "sensitises" the cell to respond to less and less potent stimuli, or even to start firing independently without external stimulation (Goddard *et al.*, 1969).

Kindling is an animal model that provides a particularly useful method for studying the nature and molecular mechanisms of fear sensitisation (Rosen & Schulkin, 1998).

Although fear responses are adaptive in that they serve as protection from harm, fear can also become sensitised so that it eventually results in behavioural responses that are maladaptive (Marks & Tobena, 1990). Accordingly, the level of fear experienced by an animal can range from what would be considered "normal" to what would be considered "pathological" (Rosen & Schulkin, 1998).

Studying the progression from normal to pathological fear is a critical approach in identifying the neural mechanisms that underlie the development of affective disorders such as generalized

anxiety and panic and in developing new pharmacological treatments that prevent or reduce these disorders. It also provides a unique opportunity to answer fundamental questions about brain sensitisation. For example, what types of behavioural responses can become sensitised? What are the neural mechanisms that underlie these forms of sensitisation? (Kalynchuk *et al*, 2001).

Kindling results in a permanent sensitisation of particular brain circuits: once an animal has been kindled (i.e. once it displays three consecutive generalized convulsive seizures), it will continue to respond to each stimulation with a generalized convulsion even after a stimulation-free period lasting several months (Goddard *et al.*, 1969). However, what should be emphasised is not the evolution of seizures per se, but the idea that kindling sensitises brain circuits responsible for behavioural manifestations of fear (Rosen & Schulkin, 1998). Thus, kindling provides both a means for inducing sensitisation and a conceptual framework from which hypotheses about sensitisation can be formulated (Kalynchuk *et al*, 2001).

Glutamate / DA pathways are responsible for inducing kindling, as animal studies signify (Post *et al.*, 1995). The basis of the kindling model is a delicate balance between GABA-ergic and glutamatergic pathways (Davies *et al.*, 1991). Very crucially, GABA-ergic pathways are pivotal to the kindling action of glutamate (Davies *et al.*, 1991), while DA is considerably involved in tolerance and sensitisation mechanisms (Post *et al.*, 1995).

Kindling phenomena have been demonstrated in limbic structures like the amygdala (Adamec, 1990), that are implicated in stress response, fear and potentially in PTSD symptoms. The mechanism of kindling may contribute to an exaggerated reaction to stressors, or consequently less stress might be needed to induce a major pathological response (Adamec & Shallow, 2000). Thus, it has been suggested that after exposure to traumatic events, limbic structures like the amygdala may become kindled or sensitized as a result of exaggerated noradrenergic input from the locus ceruleus (Post *et al.*, 1997), producing exaggerated fear responses, mood instability, anger and aggression (Albucher & Liberzon, 2002).

Thus, amygdala-kindled animals display fearful behaviour of a different nature and a greater intensity as they receive more stimulations (Kalynchuk *et al*, 2001).

Understanding the affective consequences of amygdala kindling is complicated by the fact that the first step to kindling, electrical stimulation has positive and negative motivational consequences. Amygdala stimulation provokes fear in human and animal subjects. Electrical activation of amygdala neurocircuitry previously engaged in Pavlovian fear learning may provide

useful information concerning the amygdala's role in fear reinforcement. This, in turn, may have implications for understanding the associative processes that contribute to seizure-related psychosis and other fear-associated syndromes, like PTSD (Kellett & Kokkinidis, 2004).

The results from a study by Kalynchuk *et al.* (2001) showed that inhibition within hippocampal circuits is involved in the development of kindling-induced fear. This result was expected, given that this team as well as other researchers have previously reported increased fear after amygdala kindling in rats (Kalynchuk *et al.*, 1999). Findings from the same study would also suggest that long-term kindling decreases neuronal activity and the neuroplastic capacity of the hippocampus and one of its major input structures, the perirhinal cortex. This decreased neuroplastic capacity may give rise to the abnormally high levels of fearful behaviour displayed by kindled rats after exposure to an unfamiliar open field.

With the development of the kindling model as a possible pathophysiological abnormality underlying mood oscillations, anticonvulsants were found to have anti-kindling properties, offering a possible explanation for their pharmacological effects (Weiss & Post, 1998). Consequently, drugs known to have anticonvulsant and anti-kindling effects have been considered potential treatments for PTSD (Albucher & Liberzon, 2002). Furthermore, given the promise of open-label studies with carbamazepine and valproate and the theoretical implications of such findings, Friedman (2000) is also of opinion that drugs affecting the complex processes of sensitization and kindling should be tested systematically in future drug trials for PTSD treatment .

Lamotrigine, as an anticonvulsant exhibiting the abovementioned properties, was investigated as treatment for PTSD by Herztberg *et al.* (1999). They found lamotrigine to be an effective form of treatment in PTSD, although much more extensive studies are needed in this regard.

It is in the light of this evidence that it was decided to investigate the possible anxiolytic effects of lamotrigine in the current study.

3.3 Animal models of anxiety:

There are quite a number of animal models of anxiety of which the elevated plus-maze (EPM) model will be discussed in detail.

3.3.1 The Elevated plus-maze (EPM):

3.3.1.1. Origin and early development:

The EPM test is a model based on the study of unconditioned behaviour and because it is such a pristine example, it is probably the most popular of all currently available animal models of anxiety (Dawson & Tricklebank, 1995).

This model originally derives from studies conducted on exploratory patterns, the basic premise of which was that environmental novelty simultaneously evokes fear and curiosity, thereby creating a typical approach – avoid conflict. Montgomery (1955) noted that rats consistently show high levels of exploration or preference for the enclosed alleys and concluded that, as open and enclosed alleys would evoke the same exploratory drive, the avoidance of open alleys must be due to the fact that they engender higher levels of fear. This model was further studied and redefined by Handley and Mithani (1984) and has extensively been validated pharmacologically, physiologically and behaviourally (Pellow *et al.*, 1985).

Therefore it has been accepted that normal behaviour for rodents is to stay in the closed arms. This tendency can be increased by exposing the subjects to compounds that cause heightened aversion towards the anxiety provoking open arms. However, it has also become evident that anxiolytic compounds reduce natural aversion of the open arms and would increase exploratory behaviour of the open arms (Pellow *et al.*, 1985).

It can consequently be deduced that there are determinants from the EPM that indicate less aversive behaviour and these are the number of entries made into the open arms and the time spent in the open arms (Lister, 1990).

Pellow and colleagues (1985) commented favourably on the EPM, stating that this test is:

- fast
- simple

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

Given this profile, the plus-maze would seem to offer many advantages both in routine drug screening and in the study of the mechanisms of anxiety. In this context there is little doubt that agents which modulate the functioning of the GABA receptor/chloride ionophore (e.g. benzodiazepines) produce predictable effects on anxiety as measured in this test (Rodgers & Cole, 1993).

3.3.1.2. Methodological variables:

Some methodological variables are:

- ❖ Species (It should be noted that rats and mice are the species most frequently used in EPM research) (Rodgers & Dalvi, 1997).
- With rats, the occurrence of inter-species variation is high (Pellow *et al.*, 1985).
- Age and gender.
- Housing conditions.
- Lighting levels.
- ❖ Pre-test handling.
- ❖ Time and duration of testing (Hogg, 1996).

3.3.1.3. The utility of the EPM in anxiety detection:

The effect of different stressors as well as prior stress on the behaviour of animals on the EPM was investigated in several studies.

Both Hogg (1996) and Korte and De Boer (2003) found that acute stressors, which could be forced swim and immobilisation, lead to a decreased open arm exploration.

Furthermore a study conducted by Korte and De Boer (2003) indicated the following:

- ❖ Partial immersion in water for 15 minutes neither increased nor decreased percentage time in open arms.
- ❖ Exposure to predator stress could cause a heightened state of anxiety up to three weeks post exposure.

- Anxiety instilled in mice non-pharmacologically would lead to a reduction in open arm exploration as well as a reduction in general activity and rearing.
- Rats usually prefer the closed arms and this can be enhanced by compounds that promote aversion towards the anxiety provoking open arms.
- ❖ Vehicle treated stressed rats spent less time in the open arms in comparison to unstressed vehicle treated rats.

Adamec and co-workers (2004) conducted a study which indicated that predator exposure did not increase or decrease either open or closed arm exploration.

Rodgers and Dalvi (1997) conducted a study in which conflicting results were found. They found that anxiolytic compounds reduced the natural aversion of the open arms and treatment with 5-HT_{1A} and D_2 receptor antagonists had anti-anxiety effects on the EPM. The study by Rodgers and Dalvi (1997) are supported by a study by Hogg (1996) which found results to not always be consistent with putative anxiolytic and anxiogenic compounds.

Furthermore, Rodgers and Dalvi (1997) reported that prior stress increases anxiety. This notion would be supported by the findings of an EPM study conducted in our laboratory which detected anxiety 7 days after exposure to a mild situational reminder (Naciti, 2002). It should be mentioned though that other studies have reported that re-exposure to a stressor does not influence the time spent in the open arms or number of open arm entries (Korte and De Boer, 2003).

These studies are just a few examples of the various studies utilising the EPM and gives an indication of the vast use of this animal model.

3.3.2 The open field test:

In this test emotionality is measured by defecation and locomotor activity (Lister, 1990). Gastrointestinal activity is influenced by stress and it is normal for rodents to urinate and defecate in response to stress. Emotionality can be measured in this way because the level of emotionality correlates with the amount of defecation and this measure is used along with rearing activity in the open field test (Kask *et al.*, 2002). The test is performed in a large circular arena under a high level of illumination (Lister, 1990).

This test has two main advantages: it doesn't need scores of apparatus and it is a quick procedure to perform. It is due to these advantages that this test is hugely popular and extensively used.

3.3.3 *The hole board test:*

This test offers a simple method for measuring the response of an animal to an unfamiliar environment. Previously, this test has been used to assess emotionality, anxiety and/or responses to stress in animals (Rodriguez Echandia *et al.*, 1987). Some advantages of this test are that several behaviours can be readily observed and quantified, which makes possible a comprehensive description of the animal's behaviour. However, this advantage is also a defect in that the behaviours affected by anxiety- and/or anxiogenic-relevant manipulations often vary between animals. Therefore, to overcome this problem, it is important to identify behaviour(s) of animals that are affected by anxiety and/or an anxiolytic state (Takeda *et al.*, 1998).

In this test the apparatus has holes in the floor and the test subject is allowed to poke its head through these holes (head dipping) and it is precisely this that is the measure of exploratory behaviour in this test and this may vary independently of locomotor activity (Lister, 1990).

3.3.4 *The light-dark box test:*

In 1980 Crawly and Goodwin initially proposed this test which is based on the natural aversion of rodents for brightly lit places. This test measures the test subject's partiality of the dark compartment, which is done in the following way: in a two compartment box, one compartment is dark and the other brightly lit and the total activity, the time spent in the light compartment and the crossing between light and dark compartments is measured (Oliver *et al.*, 2000).

3.3.5 *The social interaction test:*

This test measures if changes in social interaction are due to a general stimulant or sedative effect (Lister, 1990) and locomotor activity assists in this determination. The test is conducted in the following way: pairs of rats are placed in a test arena where after the time spent in active social interaction between the rats are measured. It should be noted that male rats which are unknown to each other are used (Lister, 1990). It is unfortunately the utilisation of pairs of animals which results in difficulty assessing individual behaviour (Lister, 1990).

3.4 Animal Models of PTSD:

With regard to animal models of PTSD there should be discrimination between factors that influence the manifestation of PTSD and those that are essential for induction. Yehuda and Antelman (1993) have derived the following criteria for evaluating the relevance of individual stress paradigms of the phenomenology of PTSD. These criteria were derived by pairing down PTSD to its most basic components. The four identified criteria are:

1. The stressor should have the ability to fabricate PTSD-like symptoms in a dose-dependent manner.

In the case of a stressor being of adequate severity, it should induce the biobehavioural sequel of PTSD and this results in the notion that, although duration of the stressor does not seem to be directly relevant to predicting the clinical and possibly, the biological consequences of PTSD the intensity of the stressor do play a significant role. It should be noted that there is substantiation for the idea that a relationship between stressor intensity and severity of PTSD symptoms exist. Moreover, if an animal model of PTSD is functional, behavioural and biological sequel of the stressors should have been studied in a dose-dependent manner in this model. Furthermore, in such a model, changes in relevant biological systems would show differential responsivity to different levels of similar stressors (Yehuda & Antelman, 1993).

2. The biological alterations, produced by the stressor, should persist over time or become more prominent as time passes.

It is known that the onset of PTSD is frequently postponed for months or even years in many individuals, and therefore, a steady worsening of biological abnormalities with the passage of time would be expected. It would then also be expected that a superior animal model of PTSD would theoretically allow for the prospect of recovery or attenuation of symptoms in addition to relapse or late onset. It should be emphasised that it is well established that numerous biological systems are altered directly following stress, but in many of these systems there occur a swift recovery from the stress. Therefore, the information gathered from paradigms in which animals are only studied immediately following exposure to stress can not be considered directly applicable to the biology of PTSD except if they are also followed by long-term studies (Yehuda & Antelman, 1993).

3. The biobehavioural alterations, induced by the stressor, should have the potential for bi-directional expression.

It is known that the main symptoms of PTSD contain manifestations of both enhanced and reduced responsiveness to environmental stimuli that recall the initial trauma. Consequently, it is evident that an effective animal model of PTSD should possess the ability for bipolar or bi-directional manifestations through alternations between excitatory and inhibitory biobehavioural changes (Yehuda & Antelman, 1993).

4. Inter-individual variability in response to a stressor should manifest either as a function of experience, genetics or an interaction of the two.

There exists various reasons apart form the original trauma that may lead to the induction of PTSD. It has also been well documented that not all persons who are exposed to extreme trauma develop symptoms of PTSD. As a result of these observations, many studies have examined the effects of prior stress history, family studies etc (Yehuda & Antelman, 1993).

It should be stressed that there is not any animal model, natural or induced, that can mirror the human situation in its entirety. Smith (1965) postulates that this could possibly be owed to (1) intrinsic properties with defining patient profile and diagnostic criteria, (2) inter-patient variation, (3) the level that the animal displays may not be at the level that is most pathological.

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 (Uys *et al.*, 2003). Examples include:

- ❖ Learned helplessness (Anisman, 1984).
- ❖ Stress-restress or time dependent sensitization (TDS) (Liberzon *et al.*, 1997).
- ● Exposure of animals to a predator (Adamec & Shallow, 1993).
- ● Underwater trauma (Richter-Levin, 1998).

From this selection of models the learned helplessness model (Yehuda and Antelman, 1993) and the TDS model (Liberzon *et al.*, 1997) are the most familiar and these models will subsequently be discussed, with special emphasis on the TDS model, since it was used in the current study.

3.4.1. *The learned helplessness model:*

In this model, the experimenter uses unpredictable, uncontrollable stress, in a selective manner, to differentiate between individuals with regard to escape deficit, in a following paradigm of controllable stress. Although this model was originally proposed as a model of depression, it is an acknowledged model of PTSD (Krystal *et al.*, 1989) and it has been used to form a better understanding of the potential neurobiological factors that may be involved in PTSD.

3.4.2. *Time dependent sensitization (TDS) model:*

TDS refers to the fact that one exposure to a stressor can induce an extremely longlasting alteration in the subsequent responsiveness of the organism to pharmacologic or nonpharmacologic stressors (Yehuda & Antelman, 1993).

This stress procedure involves the test animals being subjected to a brief exposure to an initial stressor with the animals being exposed to the same or another recall stressor at a later stage. Physiological and behavioural responsivity to the second stressor are significantly altered in animals previously exposed, compared with those receiving the stressor for the first time. In those instances where more than one interval between the inducing and recall stimuli have been measured, it has been shown that this effect progress with time since the first stressor. In other words, the influence of the first stressor strengthens entirely as a function of the increased passing of time (Yehuda & Antelman, 1993).

PTSD patients generally present with improved sensitivity to negative glucocorticoid feedback which is also observed in subjects exposed to the TDS procedure. If considered that subjects exposed to the TDS procedure also demonstrate distinct changes in mineralocorticoid and glucocorticoid receptor expression in the hippocampus (Liberzon *et al.*, 1999), it can be deduced that the TDS model could contribute to the study of HPA abnormalities pertinent to PTSD (Liberzon *et al.*, 1997; Liberzon *et al.*, 1999).

According to Yehuda and Antelman (1993), TDS meets al the proposed criteria for an animal model. First, it can be induced by stressful events imposed on the organism for only seconds, or, alternatively, by more chronic and severe stressors, such as inescapable shock. Such persistent TDS effects lasting at least one month have been shown after a single jab with an empty syringe needle or one injection of saline (Antelman *et al.*, 1989). Second, it is "dose dependent", having been shown after the strongest of a graded series of psychological stressors (Antelman *et al.*, 1992). Also consistent with the second criterion and as noted above,

it is induced by all types of different stressors, including physical, psychological, pharmacological and metabolic agents (Antelman *et al.*, 1991). Third, the consequences of TDS both persist for extremely long periods and, as mentioned, grow stronger with the increased passing of time; similar to what is observed in chronic or delayed PTSD. Fourth, the effects of TDS can be either excitatory or inhibitory (Antelman *et al.*, 1991), thus meeting the criterion for bipolarity. Fifth, as is the case with PTSD, TDS shows marked interindividual variability (Antelman *et al.*, 1992).

3.4.3. Predator exposure:

Adamec and Shallow (1993) exposed rats to a cat in an inescapable setting. They found long-lasting increase in anxiety-like behaviour as well as that the rodents exhibited heightened defensive behaviours for numerous hours after exposure to brief escapable exposure to a cat or cat odour. Reactions such as these in response to a recognizably life-threatening situation are reminiscent of PTSD. Adamec (1997) found that rats exposed to cats exhibited dysregulation of the HPA axis as well as an elevated unconditioned startle response with delayed habituation to startle stimuli. A further finding of this study was that human PTSD patients exhibit similar changes in startle proneness. If all the abovementioned information is taken into account, it can be deduced that the response of rodents to predator stress has characteristics similar to symptoms ensuing after stress exposure in humans.

3.4.4. Underwater trauma:

Underwater trauma in the Morris Water Maze (MWM) is a method that was first described by Morris (1984). Underwater trauma can be considered as a brief trauma due to its portrayal as a brief exposure to a life-threatening situation (Yehuda & Antelman, 1993). This model seems to have advantages such as the fact that it may be a more natural setting than other types of stressors such as electrical tail shocks as well as being easy to operate and being widely available (Richter-Levin, 1998).

3.5 Summary:

It is clear that animal models are an invaluable research tool to the investigator of anxiety and anxiety disorders. It is helpful that there is such a wide array of animal models available to assess the different aspects of anxiety and anxiety disorders and it is believed that these models will contribute greatly to the growing body of knowledge on the subject.

From this discussion of animal models of anxiety and PTSD, it is evident that the TDS model is an effective model for simulating this disorder in rodents and this explains its use in the current study. The EPM model is likewise a valuable model to assess anxiety in rodents. It is efficient and adheres to the necessary criteria for a model, validating its use in the current study.

Chapter 4: Methods and Materials

4.1 Introduction:

4.1.1. Rationale of the study:

4.1.1.1. The determination of GABA levels:

Despite the results of numerous studies investigating GABA and PTSD, the precise role of GABA in PTSD and the influence on GABA levels exerted by the stress that results in PTSD is unclear, as described in section 2.2.1.5.4. Therefore, the need for further research in this area is evident.

Vaiva *et al.* (2003) found that provided that GABA levels in the brain are genetically predetermined, the results from his study would suggest that individuals with low plasma GABA levels are premorbidly more vulnerable to stress-related disorders such as acute PTSD. If replicated, plasma GABA levels measured in the aftermath of trauma exposure might help to identify individuals at high risk for developing PTSD. This was also the first clinical study to show that psychotraumatized victims who developed PTSD at 6 weeks had a significantly lower mean plasma GABA level than those without any psychopathologic disorders.

All of the abovementioned information, but particularly the novel study conducted by Vaiva *et al.* (2003), prompted the investigation of the influence of stress induced by the TDS model on GABA levels in the current study. Although the levels could not be used as a baseline in the rats, the GABA levels at different time periods at day 1 and 7 post re-stress could be investigated.

4.1.1.2. The determination of the pharmacokinetic profiles of diazepam and lamotrigine:

It is important to relate drug levels to a pharmacodynamic (behaviour) response in pharmacological research. This is normally done by the simultaneous measurements of drug levels and an effect. In psychopharmacology different effects, such as vocalization, movement

in the elevated maze etc. can be measured. Ideally, the response should change gradually with drug concentrations, so that the entire concentration-effect curve can be determined. However, an all-or-none effect may also be used (e.g. seizure suppression). The main aim of pharmacokinetic / pharmacodynamic mathematical models is to obtain the parameters E_{max} (maximum effect) and EC_{50} (effective concentration producing 50% of the maximum effect). When these parameters are obtained the models can be used to simulate and predict drug effects under different conditions, including different disease states and treatment duration.

Anxiety is often used in the elevated maze animal model as a behaviour end point. Although these studies investigate the relationship between effect and dose, studies where the actual blood levels are measured and mathematically related to a response are limited.

One of the few studies where the actual modelling was performed was conducted by Della Paschoa *et al.* (1998). In this study fear-induced ultrasonic vocalization was used to investigate the anxiolytic effect of buspirone. This study formed the baseline for a study in which the anxiolytic effect of a novel drug, S 15535, could be compared with that of buspirone. (Vis *et al.*, 2001).

Therefore, the pharmacokinetic profiles of diazepam and lamotrigine were determined in order to establish the peak and trough levels for the drugs and to establish a pharmacokinetic / pharmacodynamic relationship for the two drugs under investigation.

4.1.1.3. The behavioural studies investigating the possible anxiolytic effect of diazepam and lamotrigine:

As described in section 1.2.1 there is no established treatment regime for PTSD. In this study diazepam and lamotrigine were investigated to determine possible anxiolytic properties of these drugs in the EPM.

Diazepam was chosen because of its well established anxiolytic effect and previous use in the EPM in our laboratory (Naciti, 2002), where it was found to be an effective anxiolytic.

Hertzberg *et al.* (1999) investigated lamotrigine as treatment for PTSD on the basis of lamotrigine being an anticonvulsant with anti-kindling properties. As described in section 3.3.2., kindling has a definite link with PTSD. The results of this study indicated that lamotrigine might be an effective form of treatment in PTSD. Based upon the findings from this study, lamotrigine was investigated to uncover possible anxiolytic effects the drug may exhibit.

On basis of the time of peak and trough levels of the drugs that were found by means of the pharmacokinetic profiles effects on aversive behaviour were measured at both peak level and trough level of each of the drugs.

4.1.2. Study objectives:

The principal aims of this project were to:

- ❖ Determine GABA levels in the hippocampus and frontal cortex of male Sprague-Dawley rats exposed to the time dependent sensitization (TDS) model as compared to unstressed control animals. GABA was determined at two times, namely 1 day post re-stress and 7 days post re-stress

- ● Construct pharmacokinetic profiles for both diazepam and lamotrigine in the rats.

- ● Investigate lamotrigine as a glutamate inhibitory anti-convulsant with anti-kindling properties and diazepam, a GABAergic anxiolytic benzodiazepine, for their possible effects in an animal model of PTSD. The pharmacokinetic profiles enabled the investigation of a relationship between the drug level and anxiolytic effect. Anxiolytic effects were determined by means of the elevated plus-maze (EPM) animal model.

4.1.3. Study outline:

4.1.3.1. The determination of GABA levels:

GABA concentrations were determined in the hippocampus and frontal cortex of rats stressed by means of the TDS procedure (n=12) and a control group which was not stressed (n=12), as discussed in section 4.5. GABA was determined both 1 day and 7 days post re-stress. This is depicted in **Figure 4-1**. GABA concentrations were determined with HPLC with EC detection.

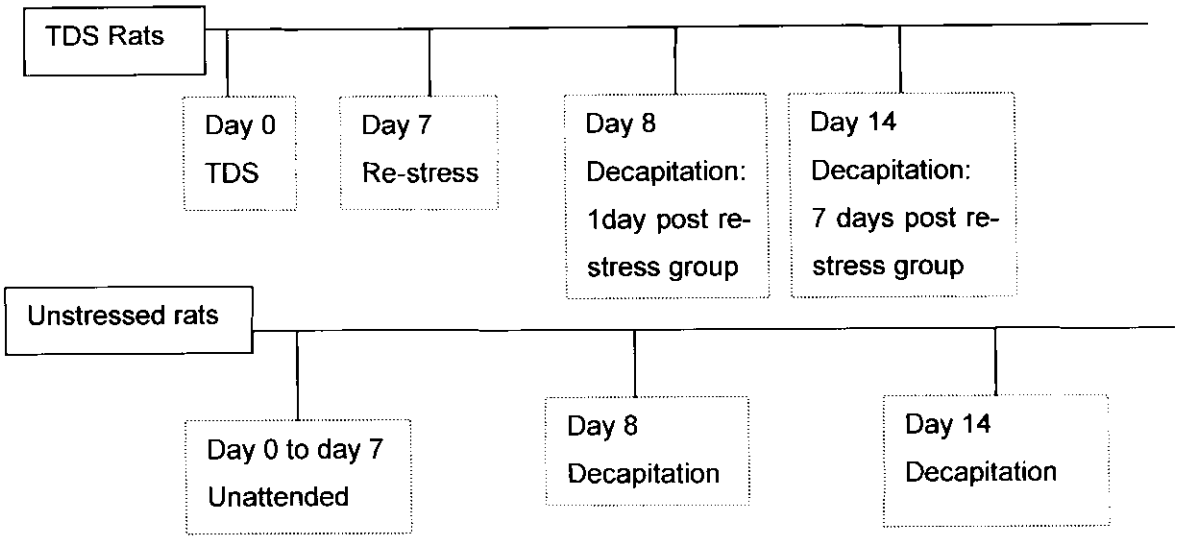


Figure 4-1: The experimental lay-out for GABA determination

4.1.3.2. The determination of the pharmacokinetic profiles of diazepam and lamotrigine:

In order to determine the pharmacokinetic profile of diazepam, rats (n=10) were injected with diazepam 3 mg/kg and from each of the rats 0.5 ml of blood was collected directly out of the heart at specified time intervals. These time intervals were 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours and 10 hours following drug administration. It should be emphasised that of these only the first six time interval's data (first 6 rat's data) were used to compile the pharmacokinetic profile, as also mentioned in section 5.3.1.

To determine the pharmacokinetic profile of lamotrigine, 3 rats were injected with lamotrigine 10 mg/kg and blood samples were taken from the tail veins at the following time intervals 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours and 10 hours following drug administration. Each rat had three specific time intervals at which a blood sample was collected. An explanation will be provided later in this section as to why different blood collection methods were used in the establishment of the two pharmacokinetic profiles.

All collected blood samples were placed on ice and transported from the animal centre to the laboratory to be centrifuged in order to obtain blood plasma. The bench top centrifuge (Sigma 3 k15) was used and blood samples were centrifuged at 5 000g for 20 minutes.

It should be noted that a maximum of 0.5ml blood could be collected at one time from the tail veins of the rats and therefore one rat could not be used to compile a complete pharmacokinetic

profile. This forced the conductors of the current study to compile data from more than one rat in order to establish the pharmacokinetic profile. It should be noted that collection from the tail veins was found to be a very time consuming exercise and therefore for the diazepam study it was decided to collect the blood samples directly from the heart. Rats do not survive this method of blood collection and therefore a separate rat had to be used for each collection time and compiled data were again used to establish the pharmacokinetic profile. The conductors of the current study are aware of the possible problematic intervariation that may have occurred with these methods of blood collection, but as is clear, the chosen method was the only effective one to the team's disposal.

The pharmacokinetic parameters were calculated by non-compartmental analyses where the observed data are used and the parameters are calculated with the following formula according to Gibaldi and Perrier (1982).

- $AUC_{(0-t)}$ The total area under the plasma concentration time curve to the last measured level by linear trapezoidal method. The formula for the linear trapezoidal method is as follows: -

$$AUC(0 \rightarrow tlast) = \sum_{i=1}^{j} (t_i - t_{i-1}) \left(\frac{C_{i-1} + C_i}{2} \right)$$

- $AUC_{(0-\infty)}$: The total area under the plasma concentration-time curve to infinity was calculated as follows: $AUC_{(0-t)} + C_n / k_e$. C_n = Last measured concentration. k_e = Terminal elimination rate constant.

❖ K_e = Log-linear regression of the last three final data points.

- $T_{½}$= The time for the concentration to decline by 50%. The half-life was calculated by: $T_{½} = 0.693/k_e$.

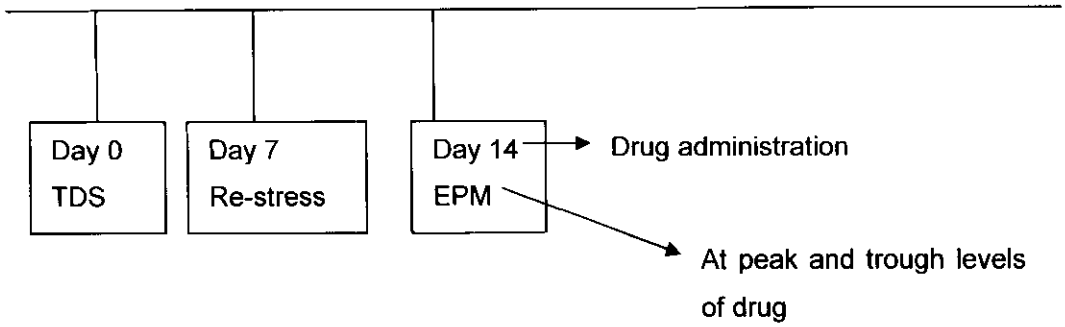
❖ C_{max}= The observed maximum plasma concentration.

❖ T_{max} = The observed time to reach maximum plasma concentration.

4.1.3.3. Behavioural studies:

The experimental lay-out followed during the behavioural studies is depicted in **Figure 4-2** and subsequently discussed:

Acute study:



Chronic study:

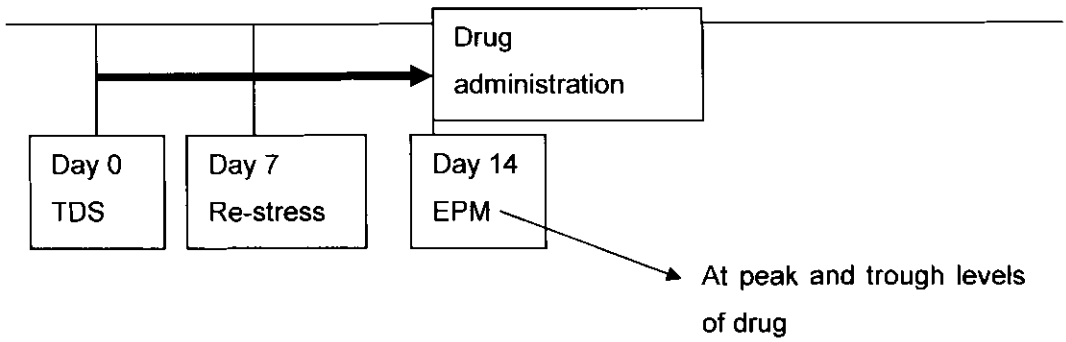


Figure 4-2: The behavioural study lay-out

This part of the study was divided into an acute study and a chronic study. It should be noted that the drugs as well as the distilled H_2O in both the acute and chronic study were administered intraperitoneally. For the acute study, all test subjects (12 per group) were subjected to the TDS procedure on day 0. All the rats were then subjected to the re-stress (forced swimming) procedure on day 7. On day 14 of the study, the day of EPM exposure, rats were administered either diazepam (3mg/kg or 5mg/kg) (n=12/group) or lamotrigine (10mg/kg) (n=12) and these groups were finally compared with their own control group of rats (n=12/group) that received only distilled H_2O. These groups of 12 were divided into 2 groups (n=6/group) with one group being subjected to the EPM at peak level of the drug and the other at trough level of the drug. The peak and trough levels of the drugs were determined by means of data from the pharmacokinetic profiles and this was also used to determine time of administration of the

drugs. Directly following the EPM exposure blood samples were collected from the rats, in the same way as for the kinetic profile determination, to be subsequently analysed by means of HPLC with UV detection. The collected blood samples were treated in exactly the same manner as the blood samples that were collected for determination of the pharmacokinetic profiles. These blood samples were collected in order to compare them to the samples collected at peak level of diazepam and lamotrigine when the pharmacokinetic profiles were established, described in the previous section.

For the chronic study, all the rats were also subjected, group by group, to the TDS procedure on day 0 and the re-stress procedure on day 7. On day 14, the same procedure was followed as for the acute study. The only difference between the studies was that for the acute study the rats were administered drugs only on day 14 and for the chronic study, rats were administered the relevant drug daily from day 0 to day 14, at approximately the same time every day, with the control groups receiving distilled H_2O at approximately the same time daily for the same period of time. Blood samples were not collected after EPM administration in the chronic study.

4.2 *Animals:*

All aspects of the study protocol was approved and performed in accordance with the guidelines stipulated by the Ethics Committee (approval number: 04D07) for the use of experimental animals at North-West University (Potchefstroom Campus). Male Sprague-Dawley rats, weighing 150-180g were used throughout the study and were provided by the Animal Research Centre of North-West University.

The test subjects were kept under the following living conditions:

- Rats were housed in identical cages (6 rats per cage). Measurements of these cages were as follows: width: 28cm, length: 44.5 cm and height: 12.5cm.
- The rats were kept under constant conditions of temperature (21±5^{0}C) and relative humidity (50±5%)
- A natural 12 hour light/dark cycle was maintained, with the light cycle in operation from 06:00 to 18:00.
- 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.

- Rodents had access to food and water ad lib.

Rats received standard rat pellets with the following composition: protein (180 g/kg), fat (25 g/kg), fibre (60 g/kg), calcium (18 g/kg), phosphor (7 g/kg) and moisture (120 g/kg).

All animals were maintained according to a code of ethics in research, training, diagnosis and testing of drugs in South Africa.

4.3 *Drugs and chemicals used in the behavioural study:*

Lamotrigine, a glutamate inhibitor, was obtained from Glaxo Wellcome Research and Development, UK. This drug was dissolved in distilled H_2O with 3 drops glacial acetic acid added and heated till dissolved.
Diazepam, a benzodiazepine was obtained from Merck Generics, South Africa. This drug was obtained as a solution in vials, ready for administration.

4.4 *High performance liquid chromatography (HPLC):*

4.4.1. *The determination of GABA levels:*

GABA concentrations in the hippocampus and frontal cortex were determined using an improved HPLC method with electrochemical detection. Joseph and Davies (1983) originally observed that fluorescent amino acid derivatives were electrochemically active. This observation provided the opportunity to combine rapidity and ease of pre-column derivatization using reverse-phase liquid chromatography with the sensitivity, selectivity and reproducibility of electrochemical detection.

GABA was determined using a modified HPLC method described by Donzanti and Yamamoto (1988). This method was validated in our laboratory by Jonker (2001).

4.4.1.1. Chemicals:

The following chemicals were used in this part of the study:

- Sodium phosphate dibasic (BDH Chemicals Ltd., Poole England).

- ❖ Disodium ethylenediamine tetraacetate (BDH Chemicals Ltd., Poole England).
- ❖ Potassium Acetate (Merck, SA).
- ● Sodium tetraborate (Rochelle Chemicals, SA).
- ❖ Methanol (HPLC grade) (BDH Chemicals Ltd., Poole England).
- ● Perchloric Acid (60%) (SAAR Chemicals, SA).
- ❖ Phosphoric acid (85%) (SAAR Chemicals, SA).
- ● Fluoraldehyde (o-Phtalaldehyde reagent solution) (Pierce, Ilinois, USA).
- ❖ Homoserine (Sigma, SA).
- ● GABA (Sigma, SA).

4.4.1.2. Chromatography :

4.4.1.2.1. Apparatus:

Stationary phase	75 x 4.6 mm Luna C18 Column (C18 reverse phase, particle size 5 µm).
Detector	GBC LC 1260 EC detector Settings: Volts = +0.600V; Range = 10 nA
HPLC-System	Agilent 1100 Series (Autosampler, isocratic pump)
Flow Rate	1.2 ml/min
Volume injected	50 µl
Temperature (Column and room)	24^0C
Guard column	3.0 mm ID x 4mm Guard Cartridge Phenomenex C18.

4.4.1.2.2. Mobile Phase:

The mobile phase consisted of the following components:

0.1M $Na_2HPO_4.2H_2O$ corresponding to 17.79g

0.13mM Na_2EDTA corresponding to 0.05g

40% MeOH corresponding to 400ml

600ml Distilled H_2O

The $Na_2HPO_4.2H_2O$ and Na_2EDTA were dissolved in 600 ml distilled H_2O after which 400 ml MeOH was added with the solution subsequently being mixed thoroughly. Following this, ±4.77 ml phosphoric acid (85%) was used to adjust the pH of the mobile phase to 6.4. Finally, the solution was degassed and filtered under vacuum through a 0.22 µm membrane filter (Millipore).

4.4.1.3. Tissue dissection and extraction :

Male Sprague-Dawley rats were decapitated and the brains rapidly removed. The hippocampi and frontal cortices were dissected and frozen in liquid nitrogen (-196°C). The frozen brain sections were then stored at -80°C until assayed.

Day before analysis:

- Approximately 10mg of the relevant brain section was taken where after it was placed in separate Pony vials.
- ❖ Subsequently 2ml of ice-cold 0.05M $HClO_4$ was added to each vial.
- ❖ These vials were sonicated for 2 x 12 seconds at 20µ.
- ❖ The vials were centrifuged at 20 000g for 15 minutes (4°C).
- The resulting supernatant was stored at -80°C until the following day.

It should be emphasized that during the preparation of the supernatant, all vials were kept on ice.

Day of analysis:

- ❖ Frozen vials were quickly thawed by putting it into 25°C water.
- ❖ Two drops of 10M CH_3COOK were added to the supernatant to obtain a pH of approximately 6.0.
- ❖ The supernatant (200µl) was added to 100µl of 5µg/ml homoserine internal standard in an amber Eppendorf vial.
- Precolumn amino acid derivitisation was accomplished by adding 450µl of the OPA reagent to the Eppendorf vial;
- ❖ The resulting solution was allowed to stand for 2 minutes before its injection into the analytical column.

4.4.1.4. Derivitization procedure:

The following procedure was performed 24 hours prior to use in order to prepare a working derivatization solution: 12,5 µl of the OPA stock reagent was diluted with 37,5 µl of 0.1N sodium tetraborate.

Derivatization was accomplished by adding 450µl of the working OPA solution to 200µl of supernatant and 100µl of 5µg/ml homoserine; the resulting solution was allowed to stand for 2 minutes before its injection into the analytical column.

4.4.1.5. Standard solutions:

A stock solution of GABA was prepared: 1 mg of GABA was dissolved in a 10 ml mixture of 5ml distilled H_2O and 5 ml methanol which yielded a solution with a concentration of 100 µg/ml. From this solution a range of the following concentrations were prepared: 5 µg/ml, 2.5 µg/ml, 1.0 µg/ml, 750 ng/ml, 500 ng/ml, 200 ng/ml, 100 ng/ml, 50 ng/ml, 10 ng/ml and 5 ng/ml.

4.4.1.6. Data analysis:

Data acquisition and analysis were performed with Chemstation Rev. A.08.03 data acquisition and analysis software. Further analysis as well as calculation of the GABA concentrations was done with Microsoft Excel (Microsoft Windows XP Professional, version 5.1) and Prism (Prism 4 for Windows, version 4.02).

The result of each experimental sample was a chromatogram. GABA concentrations were determined by dividing the peak area of each of the GABA samples by the peak area of the internal standard in the sample. This ratio was converted by means of a calibration curve of the concentration of the standard solution. The average concentration was divided by the weight (±10 mg) of the concerned brain area.

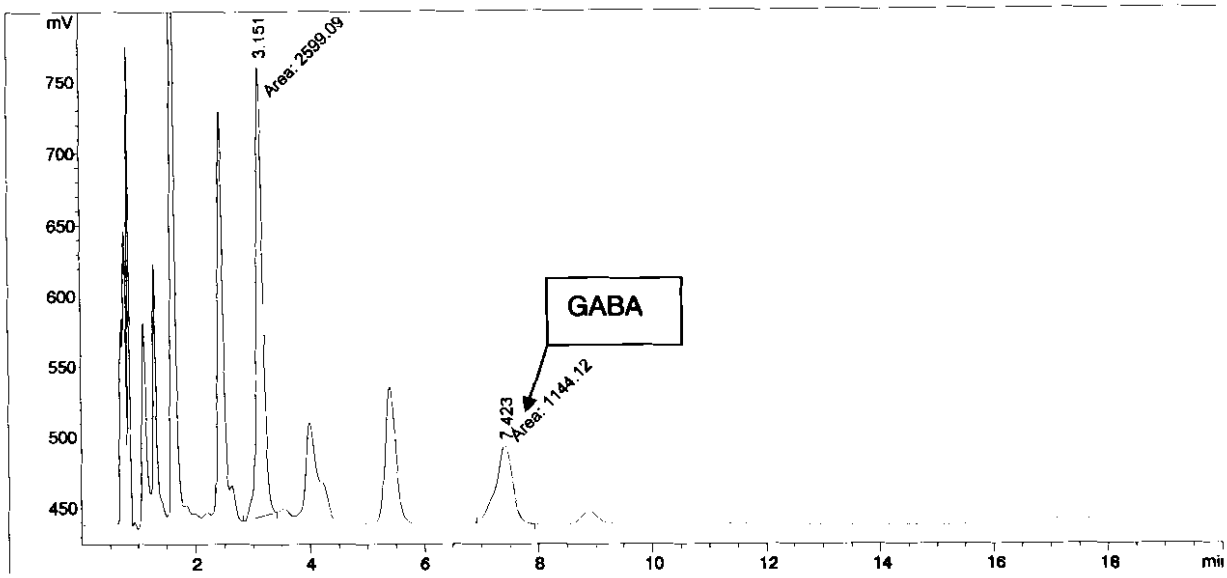


Figure 4-3: A representative chromatogram of GABA determination in the hippocampus.

4.4.2. *The determination of Lamotrigine concentrations in plasma:*

4.4.2.1. Chemicals:

- ❖ Potassium dihydrogen phosphate (Merck, Germany).
- ❖ Phosphoric acid (85%) (SAAR Chemicals, SA).
- ❖ Methanol (MeOH) (HPLC grade) (BDH Chemicals Ltd., Poole England).
- ❖ Acetonitrile (BDH Chemicals Ltd., Poole England).
- ❖ 5-(Methylphenyl)-5-phenylhydantoin (Sigma, S.A.).
- ● Lamotrigine (GlaxoSmithKline).

4.4.2.2. Chromatography:

4.4.2.2.1. Apparatus:

Stationary phase	250 x 4.6 mm Luna C18 (2) Column, 5µ
HPLC-System	Waters ™ LC module 1 Plus, Beckman System Gold Progammable Detector Module
Flow Rate	1.0 ml/min
Volume injected	10 µl
Temperature (Column and room)	25^0C

4.4.2.2.2. Mobile Phase:

The mobile phase was prepared as follows:

- 680 mg of KH_2PO_4 (Potassium dihydrogen phosphate) was weighed and dissolved in 500 ml of distilled H_2O to yield a 0.01 M KH_2PO_4 solution.
- 280 ml of Methanol (MeOH / CH_3OH) and 220 ml Acetonitrile (Methyl cyanide / CH_3CN) were added.
- The mobile phase was filtrated through a 0,22 µm filter.

4.4.2.3. Sample preparation:

- Internal standard:

To prepare the internal standard, the procedure was as follows:

1) 50 mg (or 25 mg) of 5-(Methylphenyl)-5-phenylhydantoin ($C_{16}H_{14}N_2O_2$ / MPPH) was weighed and dissolved in 50 ml (or 100 ml) of acetonitrile.
2) From the above 10 ml was taken (or 5 ml/6 ml/10 ml, in which case the peak is more visible and make up to 10 ml with acetonitrile) into a 10 ml volumetric flask.
3) The concentration obtained is = 500 µg/ml (or 125 µg/ml / 150 µg/ml / 250 µg/ml).

- Plasma samples:

Plasma samples were prepared in the following way:

1) A 100 µl of plasma was taken and 100 µl of the Internal Standard was added in a 1,5 ml Eppendorf.
2) This solution was vortexed for 20 seconds.
3) Subsequently the solution was centrifuged for 2½ minutes at 14 000 rpm in an Eppendorf 3200 centrifuge.
4) The supernatant of the sample was carried over into an insert that was placed in a vial.
5) The vial was placed into the carousel and this was placed into the Waters HPLC System.
6) 10 µl of the sample was injected by the autosampler at a wavelength of 306 nm, after the two standard samples had been injected.

4.4.2.4. Standard solutions:

These solutions were prepared as follows:

- 20 mg of Lamictin (LAM) was weighed and dissolved in 20 ml MeOH.
- 500 µl of this solution was taken and diluted in distilled water to 50 ml. This yielded a concentration of 10 µg/ml. 4 ml from this solution was taken from this 10 µg/ml solution and filled up to 10 ml with distilled water.

This solution is called the Working Standard with a concentration of LAM = 4 µg/ml.

- 100 µl of the Working Standard solution was taken and to that 100 µl of the Internal Standard was added in a 1,5 ml Eppendorf tube and vortexed for 20 seconds.
- This solution was centrifuged for 2½ minutes at 14 000 rpm in an Eppendorf 3200 centrifuge.
- *The sample was carried over into an insert that was placed in a vial.*
- The vial was placed into the carousel and the carousel was placed into the Waters HPLC system.
- 10 µl of the sample was injected by the autosampler at a wavelength of 306 nm.

4.4.2.5. Data analysis:

Apex Chromatography Workstation Software Model M625-1 (Copyright (C) 1988-1994 by Autochrom Inc.) Version 2.15 was used to capture data. Further analysis as well as calculation

of the diazepam concentrations was done with Microsoft Excel (Microsoft Windows XP Professional, version 5.1) and Prism (Prism 4 for Windows, version 4.02).

The result of each experimental sample was a chromatogram. Lamotrigine concentrations were determined by dividing the peak area of each of the lamotrigine samples by the peak area of the internal standard in the sample. This ratio was converted by means of a calibration curve of the concentration of the standard solution.

Figure 4-4 depicts a representative chromatogram of lamotrigine determination. It should be emphasised that these determinations were done on an HPLC system regularly used to determine lamotrigine blood levels for patients treated with lamotrigine. Subsequently, a patient number appears on the chromatogram, although the chromatogram indicates a lamotrigine concentration from a rat.

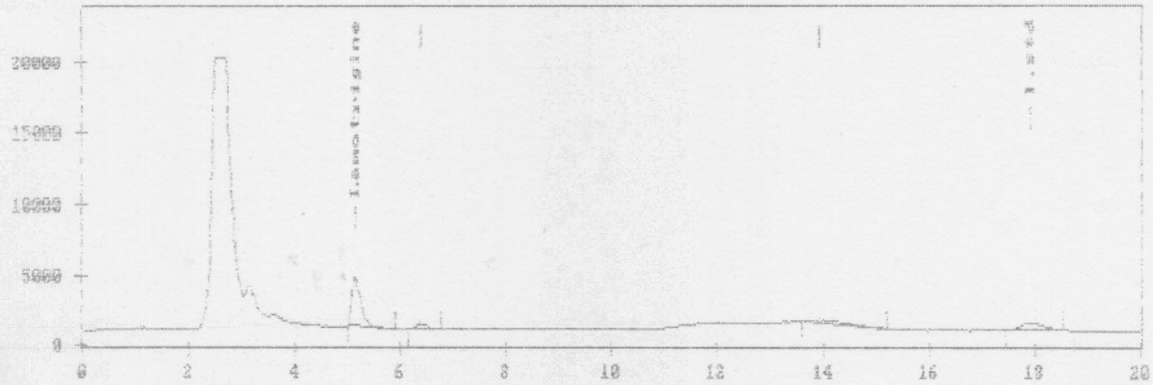
```
 Name:   Lamotrigine
Comment:  Epilepsy

cquisition method: LAMOTRIG

ample name        : Patient 17
ial ID            : 19
njection volume   : 10.00000
S amount (Samole): 250.0000
ample amount      : 1.000000
ilution factor    : 1.000000

rocessing method  : LAMOTRIG
ntegration section: created 105-01-31 14:41:01. modified 105-02-01 7:44:17
alibration section: created 105-01-31 14:41:01. modified 105-03-08 9:48:34
eporting section  : created 105-01-31 14:41:01. modified 105-01-31 14:41:01

uperior peak labels: Component Name
```



FULL REPORT

Ret Time (Min)	Component Name	Concentr.	Area (uV*Sec)	Height (uV)
5.138	Lamotrigine	3.113423	42029.5039	3509.33491
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Figure 4-4: A representative chromatogram of Lamotrigine determination.

4.4.3. *The determination of Diazepam concentrations in rat plasma:*

4.4.3.1. Chemicals:

- Diazepam (Roche).
- Nitrazepam (Roche).
- di-Ammonium phosphate buffer(Merck, Germany).
- MeOH (HPLC grade) (BDH Chemicals Ltd., Poole England).
- Glacial acetic acid (E Merck, Midrand).

4.4.3.2. Chromatography:

4.4.3.2.1. Apparatus:

Stationary phase	USP 28 (2005) packing L1 (Luna C 18-2 column, 150 x 4.6 mm, 5 µm spherical particles, 100 A pore size, 17.5% carbon loaded, endcapped, Phenomenex, Torrance, CA was used).
HPLC-System	Agilent 1100 series HPLC equipped with an isocratic pump, autosampler and UV detector (UV detection at 232 nm)
Flow Rate	1.0 ml/min
Volume injected	10 µl
Temperature (Column and room)	24^{0}C

4.4.3.2.2. Solid phase extraction (SPE) apparatus:

- A 12 positron vacuum Extraction manifold was used for the sample extraction (Phenomenex, Torrence, CA).

- Isolute 100 mg, 1 ml SPE cartridges were used (International sorbent technology Ltd., Mid Glamorgan, UK).

4.4.3.2.3. Mobile Phase:

The mobile phase consisted of a mixture of MeOH/Water/Acetic acid 70/29/1.

The HPLC grade H_2O was added to the MeOH and mixed thoroughly where after the 1% acetic acid was added. Finally, the solution was degassed and filtered under vacuum through a 45 µm membrane filter (Millipore).

4.4.3.3. Sample preparation:

- To 500 µl of blood plasma, 1000 µl of 0.1 M di-Ammonium phosphate buffer (pH 7) was added as well as 50 µl of the internal standard, Nitrasepam.
- This sample was vortexed for 15 seconds.

The prepared sample was subjected to solid phase extraction, according to the following method:

- Solid phase extraction:

1. Cartridges were conditioned with 2 ml MeOH and then with 2 ml of distilled H_2O.
2. The prepared blood samples were applied to the cartridges and allowed to pass through.
3. Cartridges were washed with 2 ml distilled H_2O.
4. Cartridges were dried under vacuum for 5 minutes.
5. Samples were eluted with 2 ml MeOH.
6. Samples were evaporated to dryness under a stream of nitrogen.
7. Samples were reconstituted in 250 µl of mobile phase.
8. Samples were vortexed for 15 seconds, transferred into inserts and analysed by means of HPLC.

4.4.3.4. Standard solutions:

Standard solutions were prepared according to the following procedure:

- 10 mg of Diazepam was added to 100 ml of distilled H_2O to yield a solution with a concentration of 100 µg/1000 µl.
- 5 ml of this solution was taken and diluted to 50 ml of distilled H_2O to yield a solution with a concentration of 10 µg/ml (Solution 1).
- 5 ml of solution 1 was further diluted to 100 ml of distilled H_2O to yield a solution with a concentration of 0.5 µg/ml (Solution 2).

In order to prepare a range of solutions with different concentrations the following procedure was followed:

Different amounts of either solution 1 or 2 were added to 500 µl blood plasma from rats that were not treated with diazepam or any other drug to result in the following concentration range depicted in **Table 4-1.**

Table 4-1: Diazepam concentration range for standard solutions.

Standard solution concentration	Amount spiked (µl)	Concentration range
0.5 µg/ml (Solution 2)	10 µl	10 ng/ml
0.5 µg/m (Solution 2)	25 µl	25 ng/ml
0.5 µg/ml (Solution 2)	50 µl	50 ng/ml
0.5 µg/ml (Solution 2)	100 µl	100 ng/ml
10 µg/ml (Solution 1)	12.5 µl	250 ng/ml
10 µg/ml (Solution 1)	25 µl	500 ng/ml
10 µg/m l(Solution 1)	37.5 µl	750 ng/ml
10 µg/ml (Solution 1)	50 µl	1000 ng/ml

The samples of the standard solutions were further prepared and analysed in exactly the same manner as the blood samples described in section 4.4.3.3.

4.4.3.5. Data analysis:

Data acquisition and analysis were performed with Chemstation Rev. A.08.03 data acquisition and analysis software. Further analysis as well as calculation of the diazepam concentrations was done with Microsoft Excel (Microsoft Windows XP Professional, version 5.1) and Prism (Prism 4 for Windows, version 4.02).

The result of each experimental sample was a chromatogram. Diazepam concentrations were then determined by dividing the peak area of each of the diazepam samples by the peak area of the internal standard in the sample. This ratio was converted by means of a calibration curve of the concentration of the standard solution.

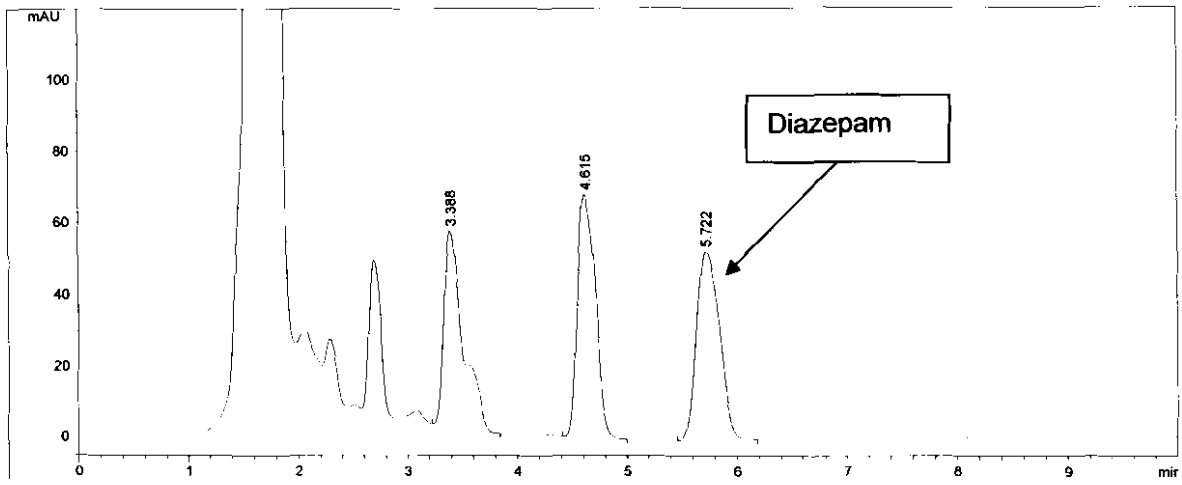


Figure 4-5: A representative chromatogram of Diazepam determination.

4.4.3.6. Validation of method:

The described method was validated in terms of linearity, accuracy and precision as well as extraction recovery.

4.4.3.6.1. Linearity:

- ❖ Preparation of standards:

These solutions were prepared as described in section 4.4.3.4.

- ❖ Acceptance criteria:

Linear regression analysis should yield a regression coefficient (R squared) of ≥ 0.99.

The response at the y-intercept sholuld be less than 5 % of the response of a 100 % standard (125 µg/ml).

The results from the current validation study adhered to these criteria with a regression coefficient (R squared) of 0.9985.

4.4.3.6.2. Accuracy and precision:

- Preparation of standards:

In order to determine accuracy and precision, standard solutions were prepared as described in section 4.4.3.4, but only solutions with the following concentrations were prepared in triplicate: 25 ng/ml, 100 ng/ml, 200 ng/ml and 500 ng/ml and a range of standard solutions with the same concentrations was also prepared. A blanko solution was prepared in the same manner but the solution was not spiked with diazepam and no internal standard was added. All of these solutions were subjected to the same procedure of sample preparation as described in section 4.4.3.3.

- Acceptance criteria:

Recovery must be between 98-102 %.

The results from the current validation study were as follows:

Table 4-2: Results from the current validation study

Sample	Sample peak area	Internal standard peak area	Peak ratio	µg/ml	% Recovered
25	43.69	504.4	0.087	37.34	149.35
25	41.75	497.57	0.084	35.82	143.27
25	42.72	491.87	0.087	37.47	149.87
100	123.73	526.87	0.235	120.49	120.49
100	107.46	440.63	0.244	125.56	125.56
100	137.29	479.17	0.287	149.48	149.48
200	222.58	490.03	0.454	243.57	121.78
500	615.39	532.88	1.155	636.62	127.32
500	595	520.54	1.143	630.01	126.00
500	595.24	512.87	1.161	639.86	127.97
25	36.37	500	0.073	29.55	118.21
100	96.6	546.94	0.177	87.83	87.83
200	211.36	538.3	0.393	209.02	104.51

500	604.45	665.1	0.909	498.60	99.72

The average percentage recovered was 125.10% (accuracy).

%RSD = Standard deviation/Average percentage recovered x 100 (Equation 4-1).

%RSD = 15.16, which is the precision.

A possible explanation for the high percentages recovered could be interference from the matrix and this interference would be especially pronounced with the lower percentages.

4.4.3.6.2. Extraction recovery:

To determine extraction recovery for diazepam and the internal standard used in the current study (nitrazepam) a diazepam solution with a concentration of 200 ng/ml was prepared in water and an internal standard solution with concentration of 50 000 ng/ml was prepared in water.

- Extraction recovery for diazepam was calculated as follows:

Peak ratio of 200 ng/ml diazepam solution in plasma / Peak ratio of 200 ng/ml diazepam solution in water x 100 / 1.

222.58 / 122.23 x 100 / 1 = 182.09%

Thus, Extraction recovery for diazepam = 182.09%.

- Extraction recovery for the internal standard was calculated as follows:

Peak ratio of 50 000 ng/ml nitrazepam solution in plasma / Peak ratio of 50 000 ng/ml nitrazepam solution in water x 100 / 1.

490.03 / 616.23 x 100 / 1 = 79.52%

Thus, Extraction Recovery for nitrazepam internal standard was 79.52%

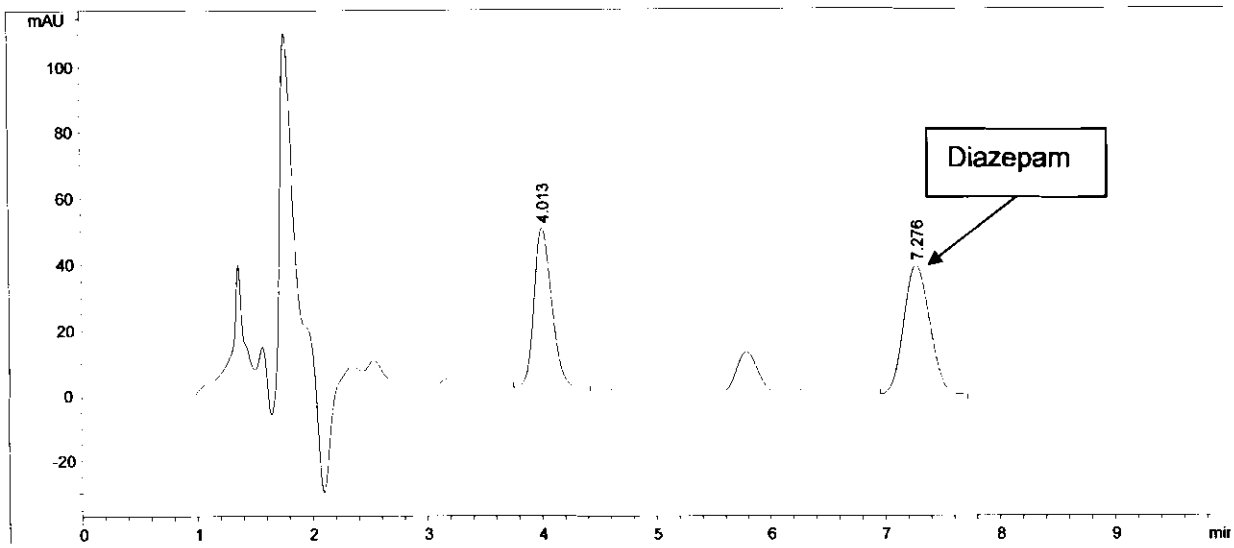


Figure 4-6: A representative chromatogram of Diazepam in the validation study

4.5 The time-dependent sensitisation (TDS) model:

4.5.1. Description and methodology:

As mentioned in the previous section, the TDS procedure implies the test subject is exposed to severe acute stress and subsequent exposure to a situational reminder of the original stress. This model is based on the notion that exposure to a situational reminder of prior stress contributes to the maintenance over time of fear-related behavioural disturbances and to the chronicity of the stress experience. In our laboratory this method has been fully validated in a study conducted by Naciti (2002).

The TDS procedure starts by subjecting the rodent to a series of repeated stressors, rapidly following each other. The first day the procedure consists of a single session of prolonged stress:

- two hour restraint in a restrainer (**Figure 4-7**).
- immediately followed by a 20 minute session of forced swimming (**Figure 4-8**).
- lastly, the animal is exposed to halothane vapours until loss of consciousness (**Figure 4-9**).

After this the animals are kept in their cage undisturbed for 6 days. The re-stress procedure commences on day 7 and consists of exposure to a single component of the initial stressor performed on day 1, in this case, an additional 20 minute forced swim (**Figure 4-10**).

It should be noted that all TDS stress sessions as well as re-stress sessions were conducted between 13:00 and 16:00.

The stress procedure is now discussed in more detail:

4.5.2.1. Restraint stress:

An individual plexiglass Perspex® restrainer is used for each rat in which the test animal is placed for 2 hours. The tailgate of the restrainer is adjusted to keep the animal well contained, without impairing circulation to the limbs.

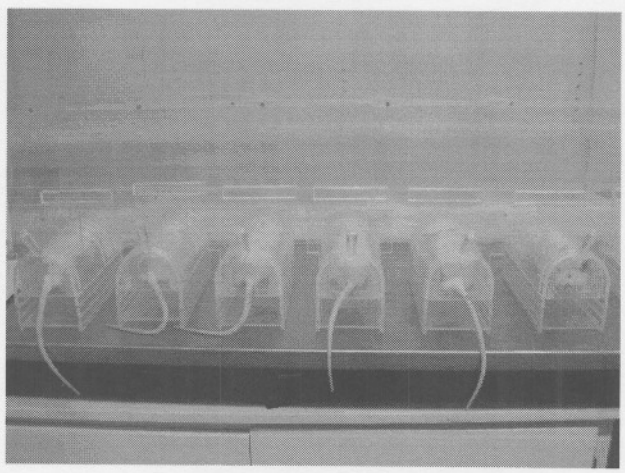


Figure 4-7: Test animals exposed to restraint stress, the first component of TDS stress.

4.5.2.2. Forced swimming stress:

Subsequent to the restraint stress each rat is placed into a Perspex® swim tank (diameter 18 cm and height 40 cm) which is filled with ambient water (25^{0}C) up to 30 cm. The animals are left in the swim tank for 20 minutes. Some observations of the rats' behaviour during this time include the following: with initial placement into the swim tank, some of the animals try to escape while others immediately start to swim. As time progress, some animals explore the swim tank by

diving down into the tank while others decrease their swimming behaviour over time with almost all animals eventually just staying active enough to keep their noses above the water. The animal is closely watched and the risk of drowning is extremely minimal.

Figure 4-8: Rats swimming for 20 minutes, the second component of TDS stress.

4.5.2.3. Halothane vapours:

After the forced swimming session the rat was hand-dried and immediately exposed to 0.7 ml of 4% halothane. The animals are individually exposed to the halothane vapours in a 5 *l* sealed plastic container until they loose consciousness, which occurs after approximately 30 seconds to 1 minute. Due to the fact that halothane vapours are very volatile and evaporate easily, an additional 0.3 ml was added after every fourth rat in order to keep a constant halothane concentration. After this procedure all animals were placed in their respective cages, which were the same cages they were housed in as groups before the TDS session and were subsequently left undisturbed until day 7, on which the re-stress procedure was performed.

Figure 4-9: The container in which rats were exposed to halothane vapour, the third component of TDS stress.

4.5.2.4. Re-stress-session:

On day 7 after the initial stress procedure (on day 0), the test animals were subjected to a situational reminder of the original stress. In the current study a 20 minute forced swimming session was used for this purpose. It should be emphasized that the conditions for the re-stress procedure were exactly the same as those of the initial forced swimming session. Subsequent to the swimming session the rats were again manually dried and returned to their cages until day 14 of the study when the EPM study was conducted.

Figure 4-10: A rat subjected to the re-stress procedure.

4.6 *The elevated plus-maze (EPM) model:*

This model was used to assess change in behaviour subsequent to the TDS procedure and is based upon a conflict between the natural aversion of rodents for open spaces and the drive to explore a novel environment.

The apparatus (made from Perspex) is depicted in **Figure 4-11** and consists of a plus-shaped maze with two open and two enclosed arms, standing approximately 0.6m above the floor. It should be noted that the open arms had a clear ledge of 0.5 cm on the edges to prevent the rat from falling off. To commence the test the rat was placed in the centre of the apparatus facing

an open arm. The animal was allowed to explore the maze for 5 minutes while a digital camera recorded the rat's behaviour for the 5 minutes. The test animal was returned to its cage after the test and the arms of the maze were cleaned with methanol to eliminate any odour-trails.

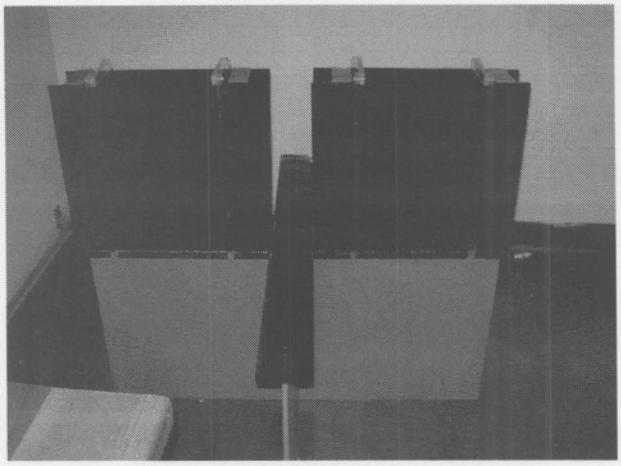


Figure 4-11: The EPM.

Analysis of the behaviour was done in the following manner: the videotaped procedures were watched in order to determine the number of open arm and closed arm entries as well as the time spent in the different arms. It should be noted that an arm entry were defined as the entry of all four feet into one arm.

These data were analysed by calculating the ratio of open arm entries and ratio time spent in the open arms.

Ratio number of open arm entries = 100 x Number of open arm entries / Total number of entries (Equation 4-2).

Ratio time in open arms = 100 x Time in open arms / Total time in both arms (Equation 4-3).

The ratio of open arm entries and ratio time spent in open arms is negatively correlated with the amount of anxiety experienced by the animal.

Locomotion, which is the total number of entries in both arms, is a non-critical parameter of anxiety as well as an indicator of motor activity (Cruz *et al.*, 1994). This parameter is calculated in the following manner:

Locomotion = total number of open arm entries + total number of closed arm entries (Equation 4-4).

4.7 Statistical analysis:

All statistical analyses were performed by Prof Faans Steyn of the Statistical Consultation Services of the North-West University.

Data resulting from the GABA concentration study were analysed using the SAS TTest procedure (SAS Institute Inc. The SAS System for Windows Release 9.1). Data from the behavioural studies were firstly analysed with a two-way analysis of variance (ANOVA) (SAS Institute Inc. The SAS System for Windows Release 9.1). Data were then further analysed with the SAS TTest procedure (SAS Institute Inc. The SAS System for Windows Release 9.1) to compare all experimental groups to the control groups as well as peak and trough groups with each other. The p-value gives the probability to erroneously reject the null-hypothesis of similar group means. If $p < 0.05$, the result is regarded to be statistically significant. All data are expressed as the mean ± Standard Error of the Mean (SEM). Prism version 4 software was used for graphic presentation.

Chapter 5: Experimental Results

5.1 Introduction:

The results obtained in the current study are presented in this chapter.

This discussion will continue in accordance with the three sections into which the study was divided:

(1) The determination of GABA levels in the hippocampus and frontal cortex on stressed and control rats at days 1 and 7 post re-stress.

(2) The pharmacokinetic profiles of both diazepam and lamotrigine in order to determine the peak and trough times and concentrations.

(3) The behavioural study to investigate the possible effect of the two drugs, diazepam and lamotrigine on aversive behaviour.

5.2 GABA levels:

5.2.1. GABA levels in the hippocampus:

GABA concentrations in the hippocampus after 1 and 7 days post re-stress can be seen in **Figure 5-1**

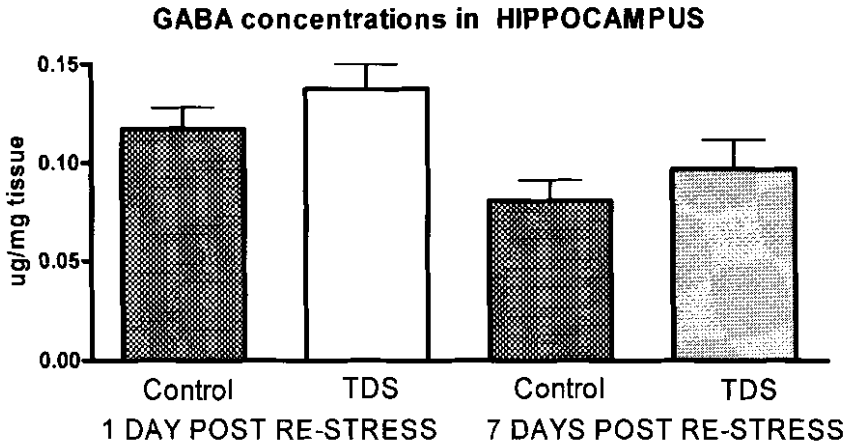


Figure 5-1: GABA concentrations as determined in the hippocampus at both one and 7 days post re-stress (mean ± SEM; n=12).

According to **Figure 5-1**, there was no statistically significant difference in concentration between control and stressed rats at both 1 day [t(21.5) = 1.23; p=0.2305; Student t-test] and 7 days post re-stress [t(13.5) = -0.89, p=0.3894; Student t-test].

5.2.2. GABA levels in the frontal cortex:

The GABA concentrations in the frontal cortex after 1 and 7 days post re-stress can be seen in **Figure 5-2**.

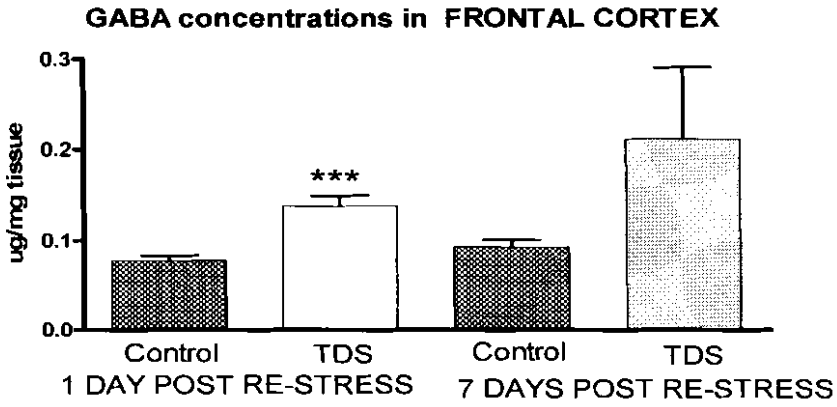


*Figure 5-2: GABA concentrations as determined in the frontal cortex at both one day and 7 days post restress (mean ± SEM; n=12), ***p=0.0002 vs Control (Student t-test).*

As depicted in **Figure 5-2**, a statistically significant increase in GABA levels was found 1 day post re-stress [t(16.9)=4.72; p=0.0002; Student t-test). At 7 days post re-stress there was a definite trend towards an increase in GABA, although this increase was not found statistically significant [t(5.1)= -1.50; p=0.1933; Student t-test].

5.3 *Pharmacokinetic profiles of diazepam and lamotrigine:*

5.3.1. *Pharmacokinetic profile of Diazepam:*

The pharmacokinetic profile in **Figure 5-3** is a combination of plasma levels from 6 rats at different time points (see section 4.1.3.2 where this procedure was described in detail).

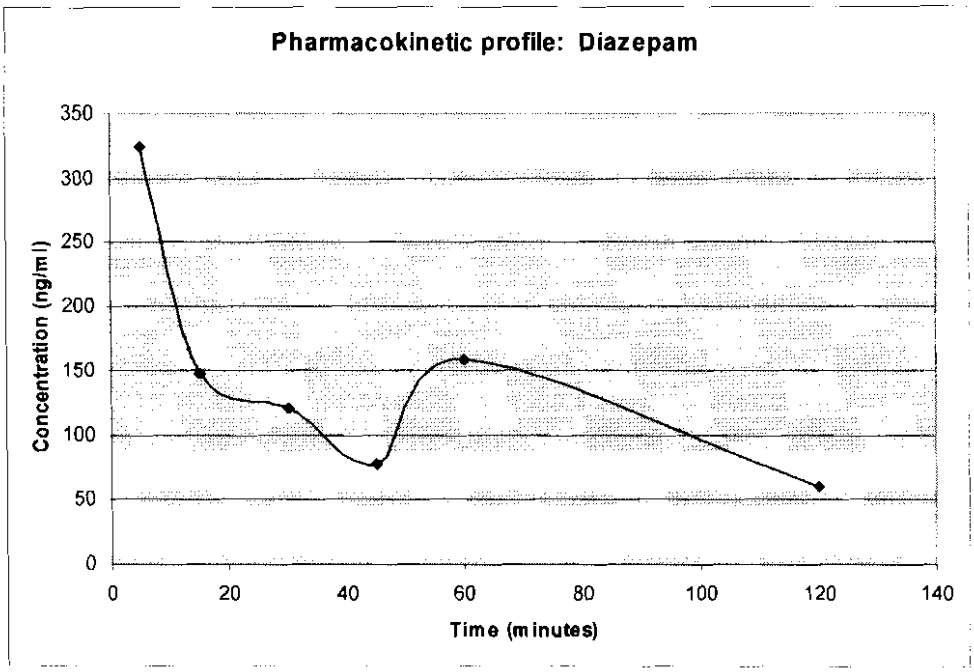


Figure 5-3: The pharmacokinetic profile of Diazepam.

The last measurable concentration was at 120 minutes due to the fact that all concentrations later than 120 minutes were below the limit of detection.

The following values were obtained from the population profile of diazepam in **Figure 5-3**:

Cmax: 159.022 ng/ml

Tmax: 1 hr

$AUC_{(0-t)}$: 250.65 $ng/ml/hr^{-1}$

$AUC_{(0-\infty)}$: 311.87 $ng/ml/hr^{-1}$

Ke: 0.98

$T_{1/2}$: 0.71 hr

From these values it can be calculated that 80% of the AUC was covered in the measured time profile.

The following concentrations were obtained at the peak level of diazepam 3 mg/kg after the test subjects were exposed to the EPM (see section 4.1.3.3 for a description of how these blood samples were obtained).

Table 5-1: Concentrations of Diazepam 3 mg/kg in 6 rats, as determined after acute administration at peak concentration of the drug, after EPM exposure.

RAT	CONCENTRATION (ng/ml)
1	135.32
2	67.01
3	67.15
4	70.17
5	66.35
6	101.47

$\bar{x}$ = 84.58 ± 28.32 ng/ml

5.3.2. *Pharmacokinetic profile of Lamotrigine:*

The pharmacokinetic profile in **Figure 5-4** is a combination of plasma levels from 3 rats at different time points (see 4.1.3.2 where this procedure was described in detail).

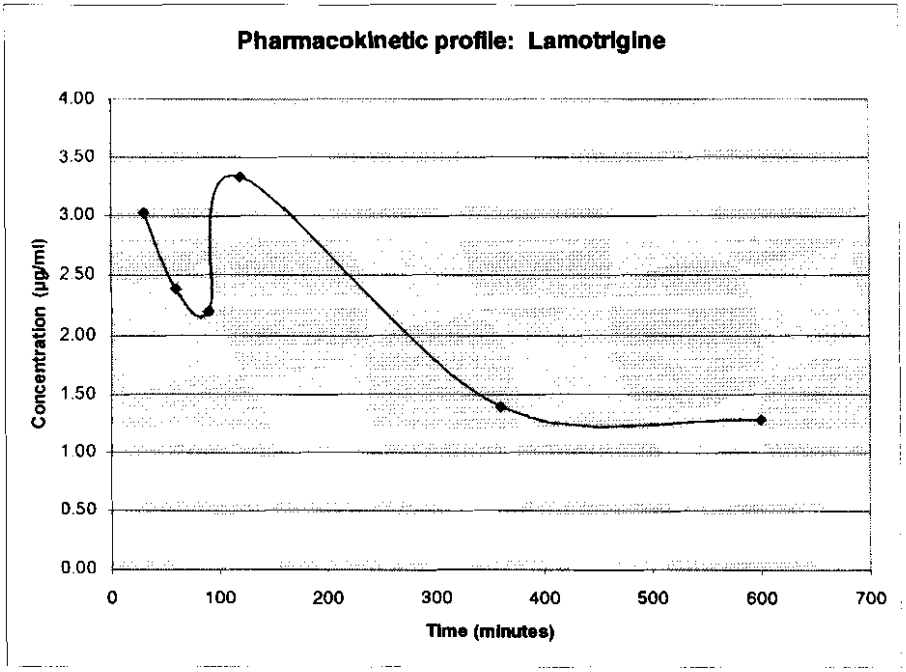


Figure 5-4: The pharmacokinetic profile of lamotrigine

The following values were obtained from the population profile of lamotrigine in **Figure 5-4**.

Cmax: 3.34 µg/ml
Tmax: 2 hr
$AUC_{(0-t)}$: 11.04 µg/ml/hr^{-1}
$AUC_{(0-\infty)}$: 63 µg/ml/hr^{-1}
Ke: 0.0206
$T_{1/2}$: 28 hr

The long $t_{1/2}$ of lamotrigine of 28 hr was the reason that only 20 % of the $AUC_{(0-\infty)}$ was covered in the time period measured. Blood samples taken at times later than 10 hours would possibly have led to more than 20 % being covered.

The following concentrations of lamotrigine 10 mg/kg were obtained at peak concentration after the test subjects were exposed to the EPM (see section 4.1.3.3 for a description of how these blood samples were obtained).

Table 5-2: Concentrations of lamotrigine 10 mg/kg in 5 rats, as determined after acute administration at peak concentration of the drug, after EPM exposure.

RAT	CONCENTRATION (µg/ml)
1	3.34
2	2.62
3	2.92
4	3.59
5	3.24

$\bar{x}$ = 3.142 ± 0.38 µg/ml

5.4 Behavioural studies:

It should be emphasized that when a control group is mentioned in the following report, this control group will refer to a group of rats that was exposed to the same stress procedure as the group they were compared to, but which did not receive the relevant drug. These control groups were only treated with distilled H_2O.

5.4.1. Results of the acute study:

5.4.1.1. Effect of Diazepam 3 mg/kg:

The effect of an acute dose of diazepam 3 mg/kg on aversive behaviour, as determined by the ratio open arm entries after EPM exposure, can be seen in **Figure 5-5**. The ratio of entries was

obtained at both the peak and trough concentrations of diazepam as determined by the pharmacokinetic profile discussed in 5.3.1.

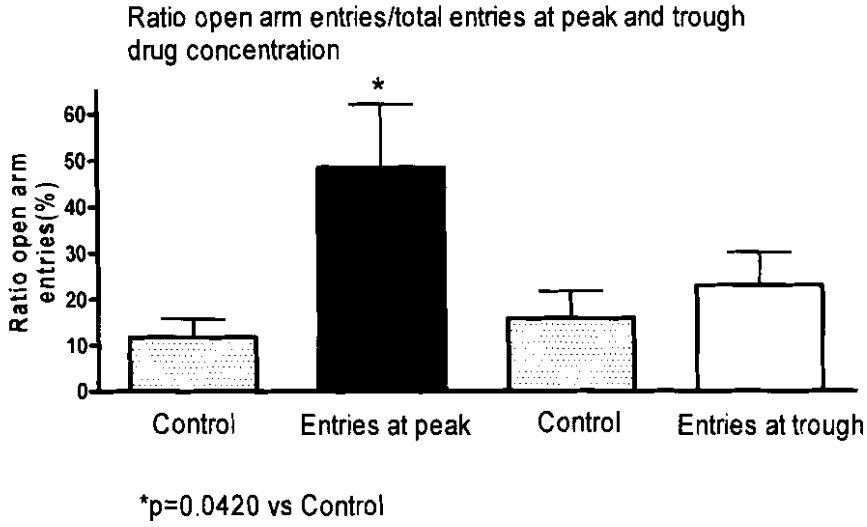


*Figure 5-5: Effect of an acute dose of Diazepam 3 mg/kg on aversive behaviour, as determined by the ratio open arm entries (mean ± SEM; n=6), *p=0.0420 vs Control (Student t-test).*

A two-way analysis of variance (ANOVA) was performed with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.0958), but the group effect was significant [F(1;20)=6.71; p=0.0175]. Data were further analysed by the SAS TTEST procedure to confirm significant differences between each of the groups and their respective control group.

The result of this t-test indicates that when rats were administered an acute dose of diazepam 3 mg/kg, a statistically significant increase (p=0.0420) in ratio open arm entries were found at the peak level of the drug, in comparison to a corresponding control group. No increase in ratio open arm entries was found at the trough level of the drug in comparison to a control group. This finding indicates that an acute administration of diazepam 3 mg/kg results in less aversive behaviour in the test subjects at the peak level of the drug.

In **Figure 5-6** the effect of an acute dose of diazepam 3 mg/kg on aversive behaviour is depicted, as determined by the ratio time spent in the open arms after EPM exposure. This ratio was obtained at both peak and trough concentrations of diazepam as determined by the pharmacokinetic profile discussed in 5.3.1.

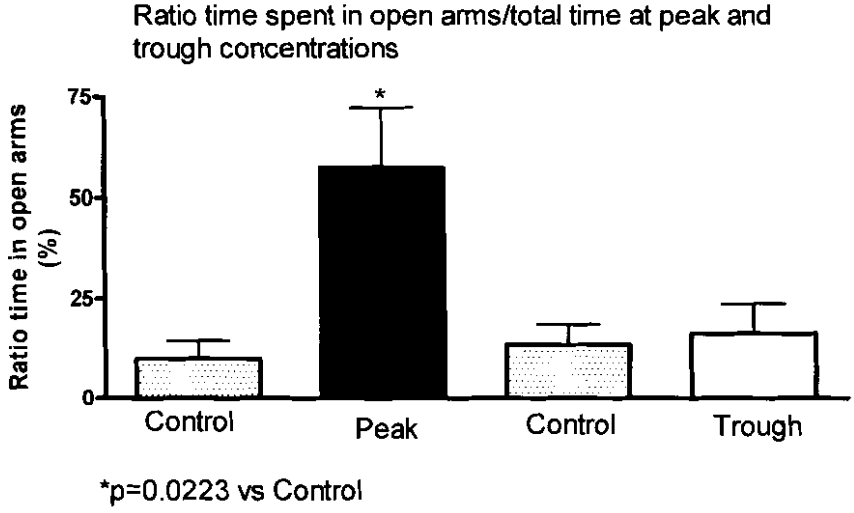


*Figure 5-6: Effect of an acute dose of Diazepam 3 mg/kg on aversive behaviour, as determined by the ratio time spent in the open arms (mean ± SEM; n=6), *p=0.0223 vs Control (Student t-test).*

A two-way analysis of variance (ANOVA) was performed with group and subgroups (peak and trough) as the two factors. A statistically significant interaction exists (p=0.0216), and a significant group effect occurs [F(1;20)=7.86; p=0.0109]. Data were further analysed by the SAS TTEST procedure to confirm significant differences between each of the groups and their respective control group.

As is depicted in **Figure 5-6**, the t-test procedure showed that acute administration of diazepam 3 mg/kg causes a statistically significant increase (p=0.0223) in ratio time spent in the open arms when compared to a control group. An increase in ratio time spent in the open arms was not found at trough level of the drug. This again indicates less aversive behaviour in the rodents at peak level of the drug, caused by an acute dose of diazepam 3 mg/kg. There was also a statistically significant difference (p=0.0402) found when the peak group was compared to the trough group, the ratio time spent in the open arms decreased from peak level of the drug to trough level of the drug.

In **Figure 5-7** the effect of diazepam 3 mg/kg as an acute dose on locomotion (calculated with Equation 4-4) is depicted.

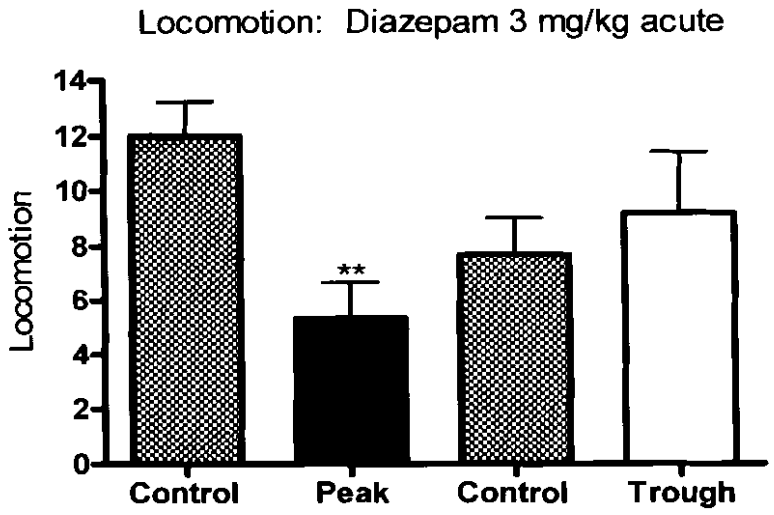


*Figure 5-7: Locomotion observed at peak and trough levels of Diazepam 3 mg/kg after an acute dose of the drug (mean ± SEM; n=6), **p=0.0039 vs Control (Student t-test).*

The effect of an acute dose of diazepam 3 mg/kg on locomotion was analysed by a two-way analysis of variance (ANOVA) with group and subgroups (peak and trough) as the two factors. A significant interaction exists (p=0.0174), but the group effect was not significant [F(1;20)=2.69; p=0.1165]. Data were further analysed by the SAS TTEST procedure.

The t-test procedure indicated that there was a statistically significant (p=0.0039) decrease in locomotion at peak level of diazepam 3 mg/kg after acute administration, when compared to a control group. At trough level, no significant increase or decrease were found in comparison to a control group.

5.4.1.2. Effect of Diazepam 5 mg/kg:

The effect of an acute dose of diazepam 5 mg/kg on aversive behaviour, as determined by the ratio open arm entries after EPM exposure, can be seen in **Figure 5-8.** The ratio of entries was obtained at both the peak and trough concentrations of diazepam as determined by the pharmacokinetic profile discussed in 5.3.1.

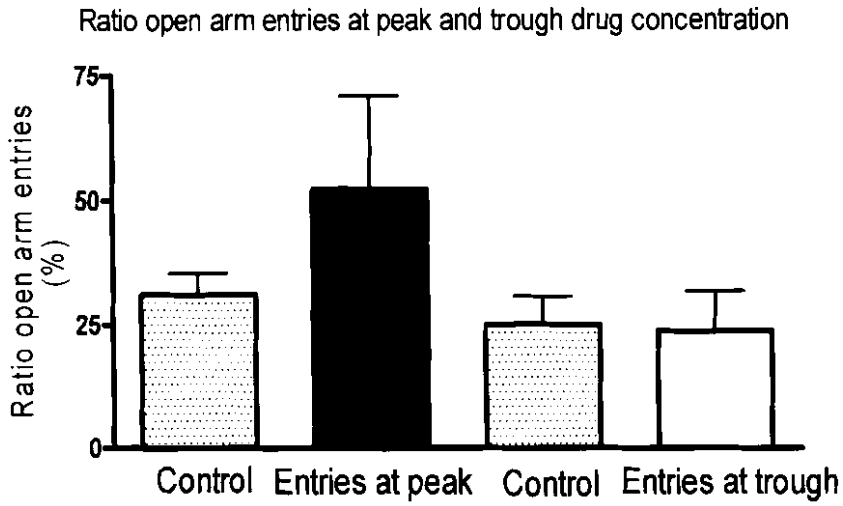


Figure 5-8: Effect of an acute dose of Diazepam 5 mg/kg on aversive behaviour, as determined by the ratio open arm entries (mean ± SEM; n=6).

A two-way analysis of variance (ANOVA) was performed with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.3065) and the group effect was also not significant [F(1;20)=0.86; p=0.3643]. Data were further analysed by the SAS TTEST procedure.

As evident from **Figure 5-8**, the t-test procedure showed that when rats were treated with 5 mg/kg diazepam, no significant difference was found in the ratio open arm entries at both peak and trough concentrations of the drug, although there was a tendency towards an increased ratio open arm entries at the peak level of diazepam 5 mg/kg.

In **Figure 5-9** the effect of an acute dose of diazepam 5 mg/kg on aversive behaviour is depicted, as determined by the ratio time spent in the open arms after EPM exposure. This ratio was obtained at both peak and trough concentrations of diazepam as determined by the pharmacokinetic profile discussed in 5.3.1.

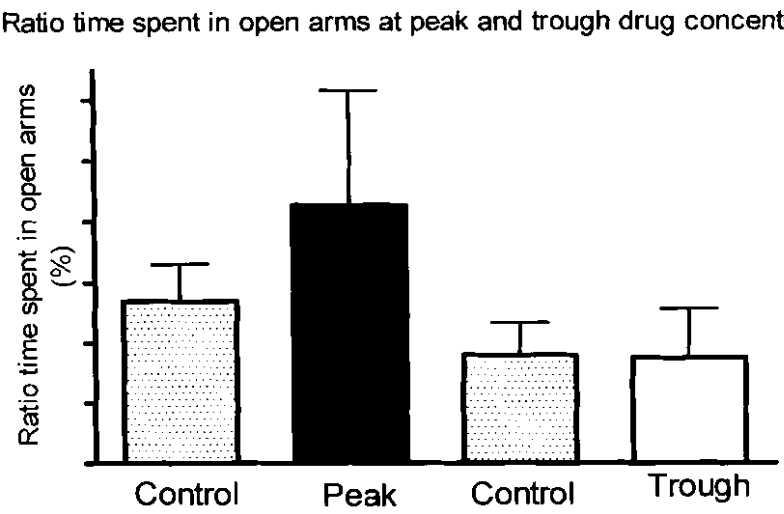


Figure 5-9: Effect of an acute dose of Diazepam 5 mg/kg on aversive behaviour, as determined by the ratio time spent in the open arms (mean ± SEM; n=6).

A two-way analysis of variance (ANOVA) was performed with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.4773) and the group effect was also not significant [F(1;20)=0.46; p=0.5032]. Data were further analysed by the SAS TTEST procedure.

As depicted in **Figure 5-9**, Diazepam 5 mg/kg as an acute dose did not result in an increase in ratio time spent in the open arms at either peak or trough concentrations of the drug.

As is evident from **Figure 5-8** and **Figure 5-9** diazepam 5 mg/kg did not have a statistically significant anxiolytic effect on the test subjects. These results may be the consequence of 5 mg/kg being to high a dose which resulted in the test subjects being sedated instead of the drug having the desired anxiolytic effect. Because of this effect of diazepam 5 mg/kg, it was decided to omit this dose in the chronic study.

In **Figure 5-10** the effect of diazepam 5 mg/kg as an acute dose on locomotion (calculated with Equation 4-4) is depicted.

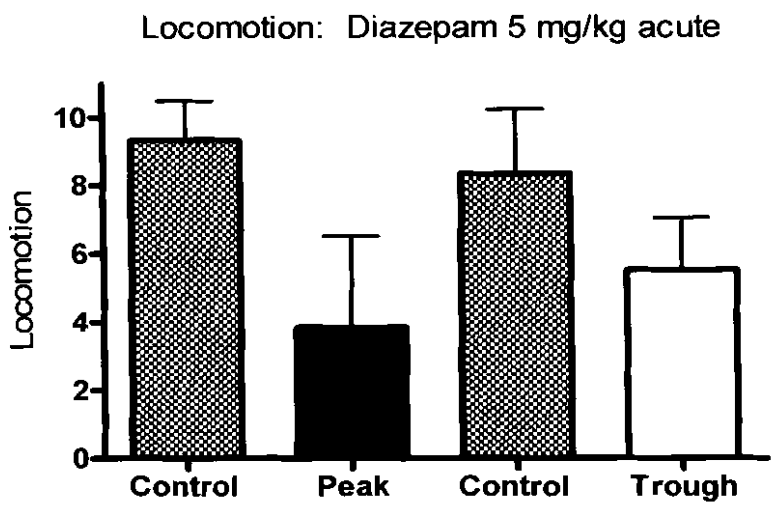


Figure 5-10: Locomotion observed at peak and trough levels of Diazepam 5 mg/kg after an acute dose of the drug (mean ± SEM; n=6).

The effect of an acute dose of diazepam 5 mg/kg on locomotion was analysed by a two-way analysis of variance (ANOVA) with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.4888), but the group effect was significant [F(1;20)=4.86; p=0.0394]. Data were further analysed by the SAS TTEST procedure.

The t-test procedure indicated that there was no statistically significant difference in locomotion at both peak and trough levels of diazepam 5 mg/kg after acute administration, in comparison to a control group.

5.4.1.3. Effect of Lamotrigine 10 mg/kg:

The effect of an acute dose of lamotrigine 10 mg/kg on aversive behaviour, as determined by the ratio open arm entries after EPM exposure, can be seen in **Figure 5-11**. The ratio of entries was obtained at both the peak and trough concentrations of diazepam as determined by the pharmacokinetic profile discussed in 5.3.1.

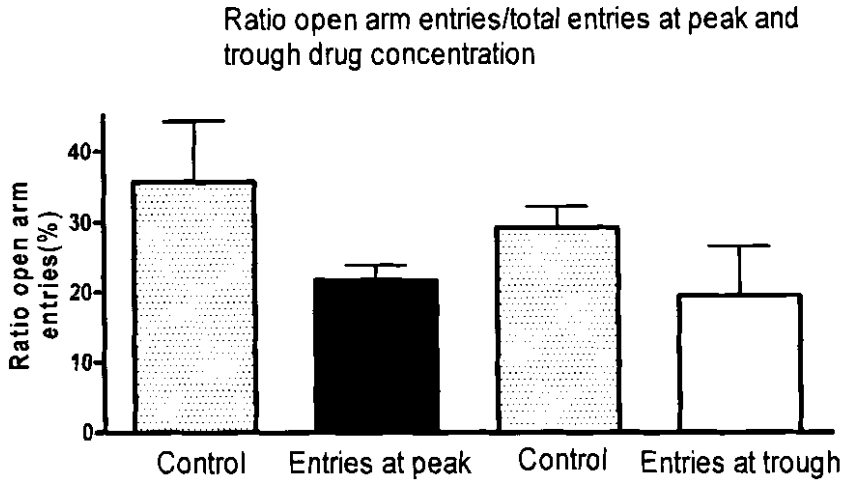


Figure 5-11: Effect of an acute dose of Lamotrigine 10 mg/kg on aversive behaviour, as determined by the ratio open arm entries (mean ± SEM; n=6).

The SAS GLM-procedure was used to perform a two-way analysis of variance (ANOVA) with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.7387) and the group effect was also not significant [F(1;18)=3.36; p=0.0835]. Data were further analysed by the SAS TTEST procedure.

As indicated by the t-test procedure and depicted in **Figure 5-11**, an acute dose of lamotrigine 10 mg/kg did not result in an increase in open arm entries, both at peak and trough concentrations of the drug.

In **Figure 5-12** the effect of an acute dose of lamotrigine 10 mg/kg on aversive behaviour is depicted, as determined by the ratio time spent in the open arms after EPM exposure. This ratio was obtained at both peak and trough concentrations of lamotrigine as determined by the pharmacokinetic profile discussed in 5.3.1.

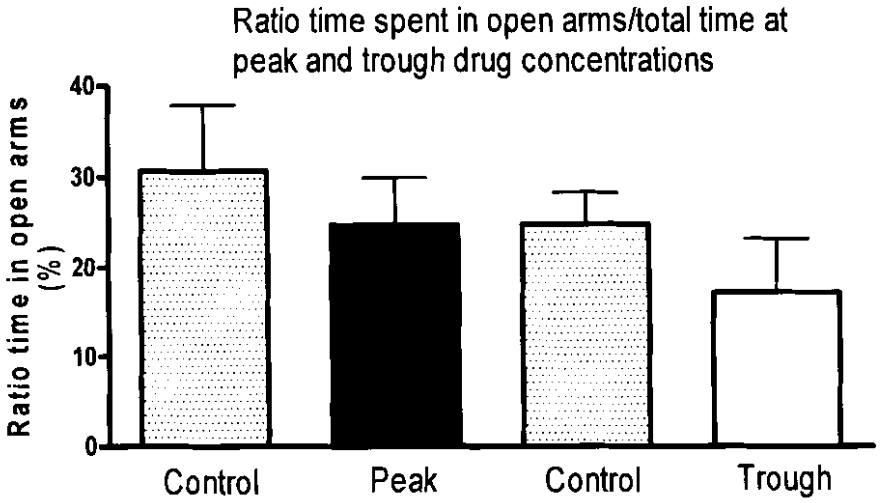


Figure 5-12: Effect of an acute dose of Lamotrigine 10 mg/kg on aversive behaviour, as determined by the ratio time spent in the open arms in EPM exposure (mean ± SEM; n=6).

A two-way analysis of variance (ANOVA) was performed with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.8953) and the group effect was also not significant [F(1;18)=1.32; p=0.2654]. Data were further analysed by the SAS TTEST procedure.

As is evident from **Figure 5-12** and indicated by the t-test procedure, an acute dose of lamotrigine 10 mg/kg does not augment the time spent in the open arms by the test subjects, either at peak or at trough concentrations of the drug.

In **Figure 5-13** the effect of lamotrigine 10 mg/kg as an acute dose on locomotion (calculated with Equation 4-4) is depicted.

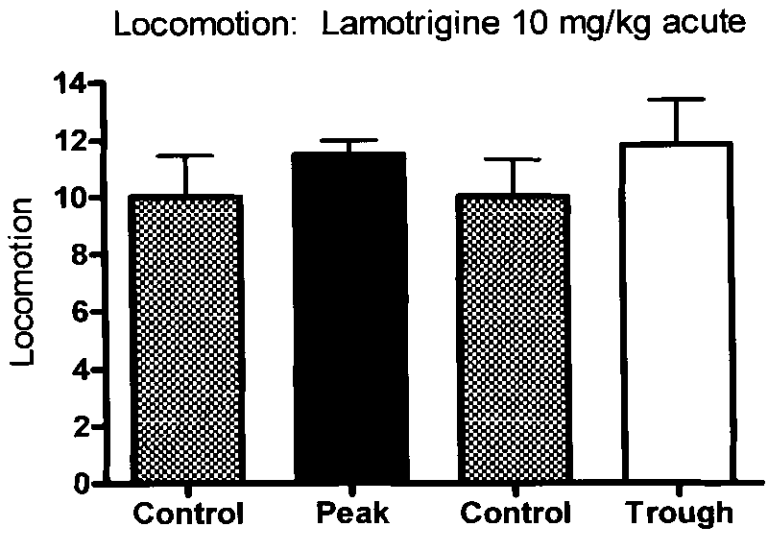


Figure 5-13: Locomotion observed at peak and trough levels of Lamotrigine 10 mg/kg after an acute dose of the drug (mean ± SEM; n=6).

A two-way analysis of variance (ANOVA) was performed with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.9070) and the group effect was also not significant [F(1;18)=1.40; p=0.2513]. Data were further analysed by the SAS TTEST procedure.

The t-test procedure indicated that there was no statistically significant change in locomotion at peak level (p=0.3682) when the group treated with lamotrigine 10 mg/kg was compared to the respective control group. A similar statistically insignificant result was found at trough level of the drug (p=0.3867).

5.4.2. *Chronic study:*

As described in 4.1.3.3, rats were treated chronically for 14 days following the TDS procedure with either diazepam 3 mg/kg or lamotrigine 10 mg/kg once daily and then subjected to the EPM procedure on day 14.

5.4.2.1. Effect of diazepam 3 mg/kg:

The effect of a chronic dose of diazepam 3 mg/kg on aversive behaviour, as determined by the ratio open arm entries after EPM exposure, can be seen in **Figure 5-14**. The ratio of entries was obtained at both the peak and trough concentrations of diazepam as determined by the pharmacokinetic profile discussed in 5.3.1.

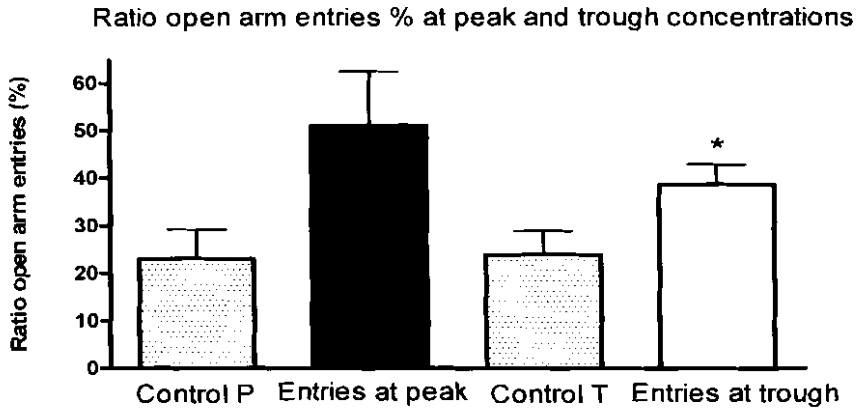


*Figure 5-14: Effect of a chronic dose of Diazepam 3 mg/kg on aversive behaviour, as determined by the ratio open arm entries (mean ± SEM; n=6), *p=0.0491 vs Control (Student t-test).*

A two-way analysis of variance (ANOVA) was performed with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.3668), while a significant group effect occurs [F(1;20)=8.61; p=0.0082]. Data were further analysed by the SAS TTEST procedure to confirm significant differences between each of the groups and their respective control group.

As is evident from **Figure 5-14**, the t-test procedure indicated that chronic treatment with diazepam 3 mg/kg did result in a trend towards an increase (p=0.0645) in open arm entries at peak concentration of the drug, although this increase was not statistically significant. There was, however a statistically significant increase (p=0.0491) in open arm entries at the trough level of the drug. This increase indicates a more sustainable anxiolytic effect for diazepam 3 mg/kg when administered chronically.

In **Figure 5-15** the effect of a chronic dose of diazepam 3 mg/kg on aversive behaviour is depicted, as determined by the ratio time spent in the open arms after EPM exposure. This ratio was obtained at both peak and trough concentrations of diazepam as determined by the pharmacokinetic profile discussed in 5.3.1.

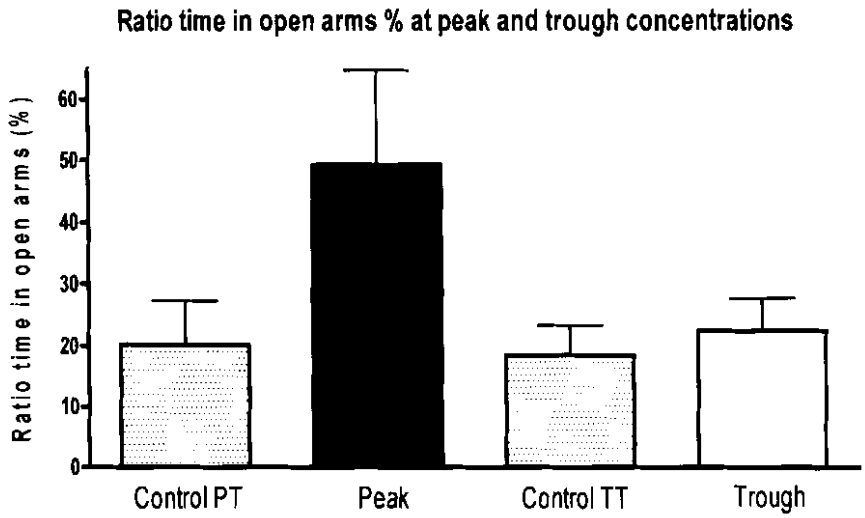


Figure 5-15: Effect of a chronic dose of Diazepam 3 mg/kg on aversive behaviour, as determined by the ratio time spent in the open arms (mean ± SEM; n=6).

A two-way analysis of variance (ANOVA) was performed with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.1907) and the group effect was also not significant [F(1;20)=3.21; p=0.0884]. Data were further analysed by the SAS TTEST procedure.

As depicted in **Figure 5-15** and indicated by the t-test procedure, chronic treatment with diazepam 3 mg/kg did not result in a statistically significant increase in time spent in the open arms, both at peak and trough concentration of the drug, but at peak concentration there was a strong tendancy towards an increase.

In **Figure 5-16** the effect of diazepam 3 mg/kg as a chronic dose on locomotion (calculated with Equation 4-4) is depicted.

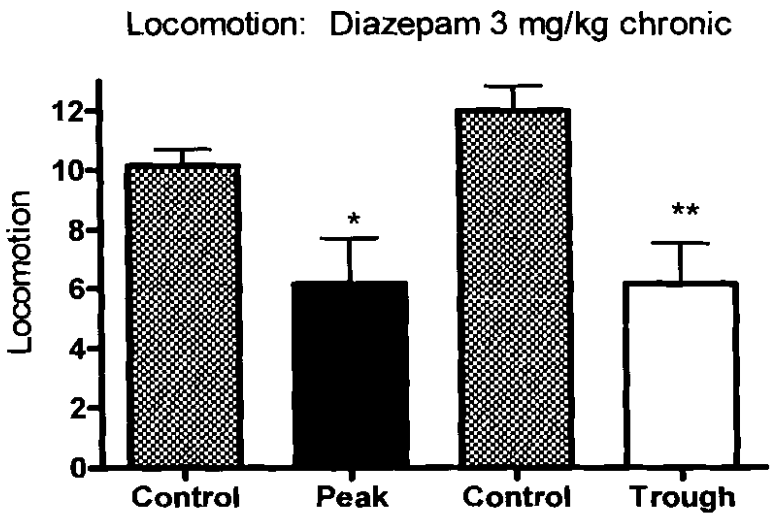


*Figure 5-16: Locomotion observed at peak and trough levels of Diazepam 3 mg/kg after a chronic dose of the drug (mean ± SEM; n=6) *p=0.0480 vs Control (Student t-test); **p=0.0064 (Student t-test).*

A two-way analysis of variance (ANOVA) was performed with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.0958) but the group effect was significant [F(1;20)=18.54; p=0.0003]. Data were further analysed by the SAS TTEST procedure.

The t-test procedure indicated that there was a statistically significant decrease in locomotion (p=0.0480) at peak level of diazepam 3 mg/kg, after chronic administration when the treated group was compared to a control group. At trough level of the drug, a statistically significant decrease (p=0.0064) in locomotion was also found when the treated group was compared to a control group.

5.4.2.2. Effect of Lamotrigine 10 mg/kg:

The effect of a chronic dose of lamotrigine 10 mg/kg on aversive behaviour, as determined by the ratio open arm entries after EPM exposure, can be seen in **Figure 5-17**. The ratio of entries was obtained at both the peak and trough concentrations of diazepam as determined by the pharmacokinetic profile discussed in 5.3.1.

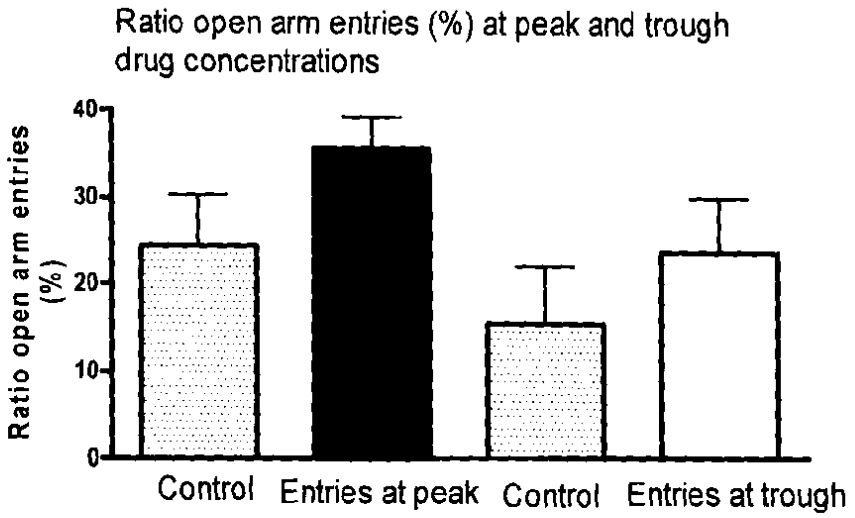


Figure 5-17: Effect of a chronic dose of Lamotrigine 10 mg/kg on aversive behaviour, as determined by the ratio open arm entries (mean ± SEM; n=6).

The SAS GLM-procedure was used to perform a two-way analysis of variance (ANOVA) with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.7840) and the group effect was also not significant [F(1;20)=2.91; p=0.1034]. Data were further analysed by the SAS TTEST procedure.

As is depicted in **Figure 5-17** and indicated by the t-test procedure, a chronic dose of lamotrigine 10 mg/kg did not result in a statistically significant increase in open arm entries, either at peak or at trough level of the drug.

In **Figure 5-18** the effect of a chronic dose of lamotrigine 10 mg/kg on aversive behaviour is depicted, as determined by the ratio time spent in the open arms after EPM exposure. This ratio was obtained at both peak and trough concentrations of diazepam as determined by the pharmacokinetic profile discussed in 5.3.1.

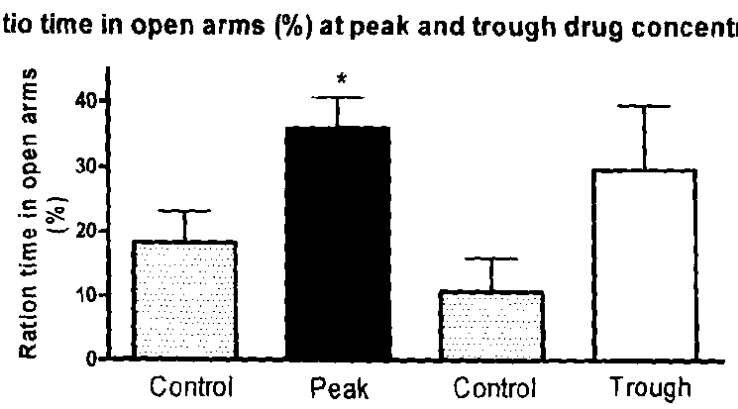


*Figure 5-18: Effect of a chronic dose of Lamotrigine 10 mg/kg on aversive behaviour, as determined by the ratio time spent in the open arms (mean ± SEM; n=6), *p=0.0262 vs Control (Student t-test).*

The SAS GLM-procedure was used to perform a two-way analysis of variance (ANOVA) with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.9054), while a significant group effect occurs [F(1;20)=7.98; p=0.0105]. Data were further analysed by the SAS TTEST procedure to confirm significant differences between each of the groups and their respective control group.

As is evident form **Figure 5-18** and shown by the t-test procedure, when lamotrigine 10 mg/kg is administered chronically, the result is a statistically significant increase (p=0.0262) in time spent in the open arms at the peak concentration of the drug. At the trough level, a statistically significant increase in time spent in the open arms did not occur, but a strong trend towards an increase was evident (p=0.1250).

This result indicates that lamotrigine at this concentration will cause less aversive behaviour in rats when given chronically and that this effect may be sustainable.

In **Figure 5-19** the effect of lamotrigine 10 mg/kg as a chronic dose on locomotion (calculated with Equation 4-4) is depicted.

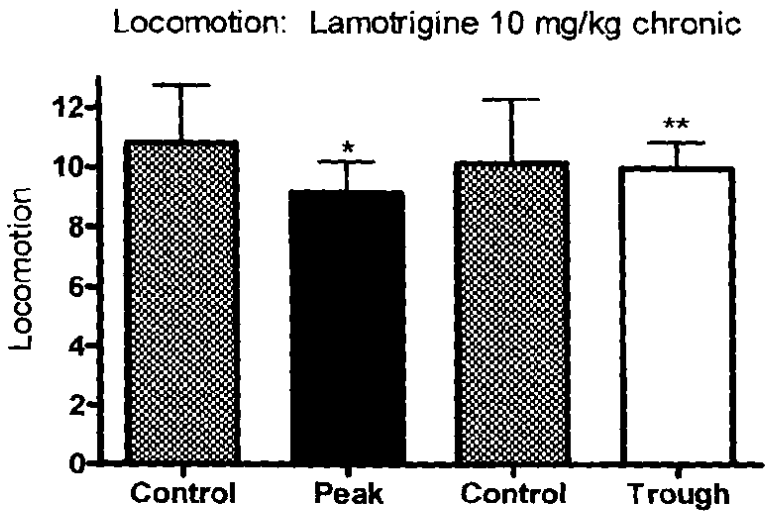


*Figure 5-19: Locomotion observed at peak and trough levels of Lamotrigine 10 mg/kg after a chronic dose of the drug (mean ± SEM; n=6) *p=0.0480 vs Control (Student t-test); **p=0.0064 vs Control (Student t-test).*

A two-way analysis of variance (ANOVA) was performed with group and subgroups (peak and trough) as the two factors. No significant interaction exists (p=0.4316), but the group effect was significant [F(1;20)=18.54; p=0.0003]. Data were further analysed by the SAS TTEST procedure.

The t-test indicated that there was a statistically significant decrease (p=0.0480) in locomotion at peak level after chronic administration of lamotrigine 10 mg/kg, as well as at trough level (p=0.0064).

5.5 Summary:

The most important results from the current study were the following:

- GABA levels were increased in the frontal cortex of rats at 1 day post re-stress following the TDS procedure and exhibit a definite trend towards an increase at 7 days post re-stress. This may indicate that this increase in GABA could be a lasting effect following the TDS procedure.

- ❖ An acute dose of diazepam 3 mg/kg results in both an increase in ratio open arm entries and ratio time spent in the open arms at the peak level of the drug. This indicates anxiolytic properties of the drug at peak concentration after an acute dose.
- ❖ An acute dose of diazepam 5 mg/kg sedated the test subjects to such an extent that the experiment could not be conducted efficiently.
- ❖ If rats were treated with diazepam 3 mg/kg for a period of 14 days, an increase in ratio open arm entries were found even at trough concentrations of the drug. This indicates a more sustainable anxiolytic effect, especially since there was a strong tendency towards an increase in ratio open arm entries at peak level of the drug.
- ● When lamotrigine 10 mg/kg were administered to rats chronically for 14 days, there was an increase in the ratio time spent by the test subjects in the open arms at peak concentration of the drug. This indicates anxiolytic properties for lamotrigine after chronic administration, supported by the finding of a strong tendency towards an increase in ratio time spent in the open arms at trough level.

Chapter 6: Discussion

The results from the current study are fascinating and can be interpreted in the following way:

6.1 The determination of GABA levels:

6.1.1. GABA levels in the rat hippocampus and frontal cortex:

GABA levels were determined in the hippocampus and frontal cortex of rats stressed with the TDS model at one day and seven days after the re-stress procedure of the TDS model. These concentrations were compared to those from unstressed rats. In the hippocampus, GABA levels were not changed statistically significantly when compared to the unstressed group, as depicted in **Figure 5-1**. This lack in change was observed at both day 1 ($p=0.2305$) and 7 ($p=0.3894$) post re-stress. As depicted in **Figure 5-2**, there was, however, a statistically significant increase in GABA levels in the frontal cortex at day 1 post re-stress ($p=0.0002$) when the stressed group was compared with an unstressed group.

In the literature no other studies could be found with the result of a lack of change in GABA levels in response to stress. However, the studies investigating a change in GABA levels post stress that were found were not in agreement on whether the response to stress is an increase or decrease in GABA. Examples of such studies are subsequently discussed. The notion that acute antidepressant withdrawal may lead to a stress response (Michelson *et al.*, 2000), prompted Harvey *et al.* (2002) to investigate the influence of such a withdrawal on behaviour and neurochemistry. Rats were treated with imipramine (15 mg/kg/day) for 21 days and then withdrawn from treatment for 7 days. The study indicated that the withdrawal stress destabilised the GABA-glutamate balance in the hippocampus and led to an increase in GABA concentrations from the decreased levels following chronic imipramine treatment to the equivalent of the levels in saline treated rats. This finding could be supported by an important finding in animal studies of TDS stress-induced activation of hippocampal inducible nitric oxide synthase (iNOS) and the down regulation of NMDA receptors being associated with a significant attenuation of total hippocampal GABA (Harvey *et al.*, 2004). GABA performs the inhibiting action it exerts on glutamatergic transmission through activation of presynaptic GABA-B heteroreceptors (Yamada *et al.*, 1999). Moreover, associated animal studies have found swim stress-induced GABA release in the hippocampus to potentiate nitric oxide (NO) (Engelman *et*

al., 2002). This points towards an important protective mechanism to curb excessive glutamate-nitric oxide synthase (NOS) activation which is exerted through GABA release. This protection is against the possible harmful effects of glutamate. However, it would appear that under conditions of repeated trauma, as with TDS, this protective effect of GABA is lost. Within this same context, it is important to note that NO also inhibits NMDA receptor-mediated GABA release (Moller *et al.*, 1995), such that raised NO depletes neuronal GABA thus leaving the exitotoxic effects of glutamate unopposed (Oosthuizen *et al.*, 2005). It could then be assumed that conditions of chronic, recurrent stress is responsible for a change the role of GABA or GABA becoming depleted, leading to GABA no longer being able to function optimally as expected. Untreated, PTSD will get progressively worse, with escalating GABA-glutamate imbalance with profound neurodegenerative consequences (Oosthuizen *et al.*, 2005).

Another study determining GABA levels in the hippocampus was conducted by Briones-Aranda *et al.* (2005). In this study Swiss Webster mice were used with the animals being sacrificed 24 hours after exposure to forced swimming for 15 minutes. The finding from this study was a decrease in GABA levels after stress.

What has indeed been illustrated by the studies mentioned in the previous paragraphs is the great variation in findings from studies investigating the effect of any kind of stress on GABA concentrations. There appears to be no agreement reached on whether GABA levels are increased or decreased by stress, with various studies mentioned supporting either notion. According to Briones-Aranda *et al.* (2005) the inconsistencies in these results seem to be due mainly to the stress factor used and the time at which the biochemical determination was made.

In the cortex, however, emotional behaviour is controlled by GABA (Davidson, 2002) and glutamate activity is also regulated by GABA. A failure to regulate and modulate heightened glutamatergic activation can therefore be detrimental, leading to extreme changes in intracellular calcium, toxicity and even cell death (Thomas, 1995). Therefore, additional GABA is released during times of stress in order to protect the brain from its own excitation (Morgan *et al.*, 2003). While glutamate and GABA are active in the PFC, this increase in GABA concentrations post stress would come as no surprise.

A study which may shed more light on the difference in the magnitude of change in GABA levels in the hippocampus and PFC was conducted by Bagley and Moghaddam (1997). They measured the change in glutamate efflux in the hippocampus and PFC after stress which was a series of three 10 minute tail pinch sessions. The findings were as follows: in the PFC, there was an initial robust increase in extracellular glutamate levels, which was similar to that

previously reported with restraint and swim stress by Lowy *et al.* (1993), Moghaddam (1993) and Moghaddam *et al.* (1994). From the first to the third tail pinch, however, there was a progressive reduction in the increase in PFC glutamate efflux in response to this repeated tail pinch. With regard to the hippocampus, the tail pinch produced a small but significant increase in extracellular levels of glutamate in the dorsal hippocampus which was smaller in magnitude than that observed in the PFC. In contrast to the PFC, an adaptation in response to repeated stress was not apparent in the hippocampus: exposure to three consecutive tail pinches produced small increases in glutamate levels that did not differ significantly between stress episodes. The finding from this study by Bagley and Moghaddam (1997) can be related to possible changes in GABA concentrations in the mentioned brain areas because as mentioned previously in this section, GABA release is increased in response to elevated glutamate levels to serve a protective function. Therefore, in response to the increased glutamate release Bagley and Moghaddam (1997) found an increase in GABA levels would also be expected. It is also expected that the increase in GABA would correspond in magnitude to the increase in glutamate and if the results of Bagley and Moghaddam (1997) are taken into account, the response in the PFC would be more pronounced than in the hippocampus, which could be related to the difference in change in GABA concentrations in the hippocampus and frontal cortex in response to stress in the current study.

An interesting study to take note of among the recent ones investigating GABA and its role in PTSD, is a study by Vaiva *et al.* (2003) which indicated that low post-trauma GABA plasma levels may be a predictive factor for the development of posttraumatic stress disorder. This finding, in combination with all the mentioned evidence for alteration of GABA after exposure to stress, may point towards an involvement of GABA in PTSD and stress that extends from before a stressful event occurs to well beyond it. The finding from the current study of a possible sustainable effect on GABA levels by stress might be an indication of this comprehensive effect of stress on GABA levels.

It is apparent that the effect stress has on GABA levels in the brain and specific brain areas, as well as the role of GABA in PTSD is still very inconclusive and avid further scientific investigation is needed in this regard.

6.2 The pharmacokinetic profiles of diazepam and lamotrigine:

6.2.1. Diazepam:

The pharmacokinetic profile of diazepam (3mg/kg) can be seen in **Figure 5-3**. The plasma levels were generated from plasma samples of 6 rats and the main objectives were to establish peak and trough concentrations for diazepam as well as the time of these peak and trough concentrations in the current study population.

Although the profile didn't provide for interindividual variation, certain observations could be made which correspond with data from the literature.

In the graph (**Figure 5-3**) two peaks were observed at times 5 and 60 minutes. The two-peak phenomenon was also documented by Wang *et al.* (1999).

Several mechanisms have been proposed for the double-peak phenomenon that was found in the pharmacokinetic profile; viz.: 1) enterohepatic recycling, 2) the presence of absorption windows along the gastrointestinal tract and 3) variable gastric emptying (Wang *et al.*, 1999). Wang *et al.* (1999) hypothesizes that the double-peak phenomenon that occurred in their study of alprazolam may be due to reduction in gastric motility caused by the muscle relaxant effect of alprazolam. Wang *et al.* (1999) further states that benzodiazepines, such as diazepam, have been found not only to relax airway muscle by a direct action on airway smooth muscle in guinea pigs, but also to alter the gastrointestinal motility in conscious dogs. Therefore it can be deduced that diazepam could also have the same effect on gastric motility in rats which may lead to a double-peak phenomenon in a pharmacokinetic profile.

Diazepam obeyed two-compartment pharmacokinetics and therefore the second peak was observed as the peak level of diazepam. At the peak level of the drug, the blood of 6 individual rats was obtained. The half-life from our data was calculated as 0.71 hours which correspond with a half-life of 1.1±0.2 hours calculated by Klotz *et al.* (1976). The fact that 80% of the AUC was covered in 2 hours strengthened the observation of a short half-life.

From the current study and the literature it can be concluded that the $t_{1/2}$ of diazepam in rats is shorter than in humans (1 vs 28 hours) (Klotz *et al.*, 1976) and the effect of the drug will be shorter. This was demonstrated in the current study when the EPM at peak time indicated a statistically significant increase in both ratio open arm entries and ratio time spent in the open arms after acute administration to rats of diazepam 3 mg/kg as well as by the finding of a

statistically significant decrease in ratio time spent in open arms from peak level of the drug to trough level (these results will be discussed further in section 6.3.1.1). The statistically significant difference in the EPM parameter ratio time spent in open arms at peak and trough times after acute administration also strengthened the finding of a difference in concentration.

6.2.2. *Lamotrigine:*

The pharmacokinetic profile for lamotrigine (10 mg/kg) can be seen in **Figure 5-4**. The plasma levels were generated from 3 rats, as described in section 4.1.3.2.

The peak concentration in this study was 3.34 µg/ml at 2 hours. The concentration of 3.142 µg/ml corresponds with a value of 2.9 µg/ml at 2 hours reported by Walker *et al.* (2000) and the average concentration of 3.142 µg/ml reported in the current study which was calculated from the lamotrigine concentrations of 5 rats determined after EPM exposure at peak level of the drug.

The half-life calculated in the present study was 28 hours; the long half-life was confirmed by the fact that only 17.53 % of the AUC was covered at 10 hours. A half-life longer than 30 hours was also documented by Walker *et al.* (2000).

The trough level of lamotrigine was found at 6 hours and this indicates very slow elimination. This characteristic of lamotrigine was also observed by Walker *et al.* (2000). In the study by Walker *et al.* (2000) it was found that lamotrigine speedily appears in serum post intraperitoneal administration, which indicates ready penetration from the peritoneal cavity. In this study, the serum pharmacokinetics were found to be biphasic; the first phase expressing distribution from the blood compartment and the second phase demonstrating principally elimination. In the rat an extended elimination phase was found. This may be a result of the inability of the rat to efficiently conjugate lamotrigine (Dickens *et al.*, 1995). These kinetic effects would predict a prolonged action of lamotrigine in rat models of epilepsy, and are important in the determination of lamotrigine's effects in chronic animal models such as kindling (Walker *et al.*, 2000).

The results from the behavioural study performed in the current project underlined this observation of a small difference between peak and trough concentrations. When lamotrigine 10 mg/kg was administered to rats as an acute dose, no statistically significant increase in ratio open arm entries or ratio time spent in the open arms in response to the drug treatment was found either at peak or at trough levels of the drug. After chronic treatment, however, a

statistically significant increase in ratio time spent in open arms was found at the peak level of the drug and this finding corresponds with the long half-life of lamotrigine.

6.3 Behavioural studies:

The EPM was used as behavioural model and three different parameters were analysed to get an indication of the drugs' effects on aversive behaviour, viz. ratio open arm entries, ratio time spent in open arms and locomotor activity (see section 4.6).

6.3.1. Results of the acute study:

6.3.1.1. Effect of diazepam 3 mg/kg:

Diazepam 3 mg/kg was found to cause less aversive behaviour in rats, this finding is based on the fact that an acute dose of diazepam 3 mg/kg resulted in an increase in ratio open arm entries ($p=0.0420$) as well as ratio time spent in the open arms ($p=0.0223$) at peak level of the drug versus control as depicted in **Figure 5-5** and **Figure 5-6** respectively. As is evident from these figures, there was no significant increase in either of these two parameters at trough level of the drug.

Also depicted in **Figure 5-6** is a statistically significant decrease ($p=0.0402$) in ratio time spent in open arms if this ratio at peak level of the drug is compared to this ratio at trough level of the drug. This finding is in accordance with findings from the pharmacokinetic profile of diazepam which also indicates a big difference between effect at peak level and trough level. This difference could be explained in the following manner: after an acute dose of the drug the $t_{1/2}$ of diazepam in rats was found to be to short in order for steady state concentrations to be reached and therefore the drug's effect differed hugely between peak and trough concentrations. Thus, the lack of an anxiolytic effect at trough level of the drug could also be explained by the mentioned pharmacokinetic findings. With acute dosing, steady state was not obtained in one dosing interval and the difference in concentrations was reflected in the pharmacodynamics.

The finding in the current study of diazepam 3 mg/kg resulting in an increase in both ratio open arm entries and ratio time spent in the open arms at peak level of the drug, after being administered as an acute dose is supported by numerous studies, subsequently mentioned.

Firstly, it should be noted that the EPM model used in the current study was validated in our laboratory (Naciti, 2002). Although the EPM apparatus used in the current study were fitted with ledges, which were absent in the study by Naciti (2002), all other experimental conditions were the same. For the validation study a range of different concentrations of diazepam were administered to rats. Of these concentrations 3 mg/kg was the only one found to increase the ratio open arm entries statistically significantly and therefore caused the most pronounced decrease in aversive behaviour.

These results are in agreement with a study conducted by Dunn *et al.* (1998), where it was found that diazepam 3 mg/kg exhibited an anxiolytic effect in rats, as determined by the EPM. Further support for the findings from the current study is found in two more studies conducted in rats. The first of these were conducted by Gomez *et al.* (2001) in which the rapid anxiolytic activity of progesterone and pregnanolone were compared with the anxiolytic effect of diazepam. In this study it was again found that diazepam exhibited an anxiolytic effect according to the EPM. In the second study De Almeida *et al.* (2004) compared the anxiolytic-like effect of rose oil inhalation on the elevated plus-maze to diazepam (1 mg/kg and 2 mg/kg). This study also found diazepam to exert an anxiolytic effect.

A number of studies conducted in mice also support the finding in the current study of diazepam 3 mg/kg leading to less aversive behaviour in rats at peak level of the drug after an acute dose. Firstly, the results from a study conducted by Dalvi & Rodgers (1996) indicated that diazepam (1.5 mg/kg) caused DBA/2 mice to enter the open arms of the EPM more frequently. These results subsequently led Dalvi and Rodgers (1996) to the conviction that this pattern of behavioural changes is typical of benzodiazepine anxiolytics under the relevant test conditions and are consistent with reductions in anxiety, conflict and risk assessment, together with a concomitant stimulation of exploratory behaviour. It should be emphasized that Dalvi and Rodgers (1996) administered the diazepam in their experiment as an acute dose 30 minutes before the test subjects were subjected to the EPM, a procedure which is similar to the procedure adhered to in the current study. A similar study was performed again by Dalvi and Rodgers (1999) in which the results of the previously mentioned study were repeated.

Two further studies conducted in mice were performed by and Jain *et al.* (2005) and Clenet *et al.* (2006). Firstly, Jain *et al.* (2005) found diazepam (0.5 mg/kg, intraperitoneally) to reverse the caffeine induced decrease in both open arm entries and time spent in the open arms; diazepam reversed the anxiogenic effect of caffeine. Secondly, the study by Clenet *et al.* (2006), conducted with Swiss mice, highlighted diazepam's stimulatory effects on exploration illustrated

by the augmentation of both the number of open and closed arm entries in the EPM. The anxiolytic effects of diazepam seem to be better demonstrated in terms of open arm entries as opposed to time spent in the open arms (Clenet *et al.*, 2006). It should be emphasized that in the mentioned studies, diazepam was administered as an acute dose 30 minutes before the test subjects were subjected to the EPM, a procedure which is similar to the procedure adhered to in the current study.

There is no doubt that diazepam has anxiolytic effects in numerous studies done in various species of test animals in which a wide array of animal models were used. In the current study the effect of different drug concentrations at different time periods were also investigated. All of these studies are in agreement: diazepam is highly effective in decreasing aversive behaviour in the test animals. This is true to such an extent that diazepam has been called the anxiolytic reference drug (De Almeida *et al.*, 2004).

The third parameter that was analysed, locomotor activity, was however decreased following diazepam 3 mg/kg as acute dose at the peak level of the drug. This is however not a clear indication that diazepam does not lead not less aversive behaviour in rats. Cruz *et al.* (1994) found in their study that total number of entries was a mixed index of both anxiety and motor activity. Therefore, this finding in the current study could be indicative of a possible effect of diazepam on motor activity and not anxiety/aversive behaviour. The finding in the current study and this notion is supported by a study by Aburawi *et al.* (2003) where it was found that triazolam exerted an anxiolytic effect while decreasing locomotion. According to these authors this depression of motor activity is a clear indicator of the predominance of sedation and probably muscle relaxation. As mentioned earlier in this section, a result of diazepam 3 mg/kg causing less aversive behaviour in rodents at peak concentration of the drug after acute administration was found in the current study. It is then interesting to note that the effect an acute dose of diazepam 3 mg/kg was found to exert on locomotion in the current study was also found just at the peak concentration of the drug.

Although the benzodiazepines are not first-line therapy in the treatment of PTSD (Wells *et al.*, 2003) a few clinical studies indicated that these drugs may be of value in reducing PTSD symptoms.

In one such study trauma survivors with an acute stress disorder were administered benzodiazepines for sleep over the short-term and they found this treatment was associated with an acute reduction in PTSD symptoms (Mellman *et al.*, 1998). Another study conducted by Braun *et al.* (1990) found alprazolam, a benzodiazepine, to decrease overall anxiety ratings,

although the study didn't find a reduction in core PTSD symptoms in reaction to the alprazolam treatment. In this study alprazolam (3.75mg) was compared to placebo in a 5-week cross-over design, with a two week washout between drug and placebo phases.

6.3.1.2. Effect of diazepam 5mg/kg:

The results from this treatment regime are depicted in **Figure 5-8**, **Figure 5-9** and **Figure 5-10**

As mentioned in the previous section, diazepam was expected to exert an anxiolytic effect. In the current study however it was found that this dose exerted a sedating effect so overpowering, the anxiolytic effect could not be detected. The test subjects were sedated to such an extent that in certain cases no entries were made, the subject, in its sedated state, remained in the centre of the EPM, where it was placed right at the start of the procedure. This finding led to the decision not to use the dose of 5mg/kg in the chronic study.

In the validation study conducted by Naciti (2002), it was also found that this dose of diazepam had a sedative effect. Moreover, it was found that this sedative effect caused the animals to be less mobile, a finding which concurs with the finding of the current study.

6.3.1.3. Effect of lamotrigine 10 mg/kg:

The current study did not indicate that lamotrigine 10 mg/kg, as an acute dose, cause less aversive behaviour in rats as depicted in **Figure 5-11** and **Figure 5-12**, however there was a statistically insignificant trend toward a decrease in both ratio open arm entries and ratio time spent in open arms. This trend was observed at both peak and trough levels of the drug. As mentioned in section 6.2.2, lamotrigine is eliminated very slowly and this would explain the similarity in effect at peak and trough levels of the drug.

The finding of an acute administration of lamotrigine 10 mg/kg not leading to less aversive behaviour in rats at any level of the drug could be explained by the fact that steady state levels of the drug have not been reached after only an acute dose and steady state or higher levels of the drug is needed to exert this effect. This observation is supported by a clinical study conducted by Hertzberg *et al.* (1999). In this study lamotrigine was found to be an effective form of treatment in PTSD after the test population was subjected to a chronic treatment regime. In further support of this notion, it should be mentioned that the current study did yield a result of increased ratio time spent in the open arms of the EPM at peak concentration of lamotrigine 10

mg/kg after chronic administration of the drug for 14 days. This result will be discussed in section 6.4.2.2.

It should also be noted that lamotrigine 10 mg/kg, as an acute dose, had no significant effect on locomotion in the rats, although there was a trend towards a slight increase in locomotion at both peak (p=0.3682) and trough levels (p=0.3867) of the drug. The lack of lamotrigine exerting a significant effect and only exhibiting a trend could be a further indication of the drug not being able to exert a full effect due to inadequate drug levels.

6.3.2. *Results of the chronic study:*

Chronic drug administration entailed the rats being injected intraperitoneally with the drug or distilled H_2O once daily for 14 days, commencing immediately following the TDS exposure, as described in section 4.1.3.3.

6.3.2.1. Effect of diazepam 3 mg/kg:

As depicted in **Figure 5-14**, the finding of diazepam 3 mg/kg, administered for 14 days, causing less aversive behaviour at trough level of the drug (p=0.0491), as well as showing a strong trend towards less aversive behaviour in rats at peak level (p=0.0645), indicates that the anxiolytic effects following chronic treatment is more sustainable than the anxiolytic effect of diazepam 3 mg/kg after an acute dose, which did not indicate an anxiolytic effect at trough level. This sustainability of effect is expected because after chronic administration of the drug, steady state drug levels would be reached and a significant difference between effect at peak and trough levels would not occur. It is also expected that because steady state drug levels were reached after chronic administration of the drug there would occur a significant effect at peak level of the drug and therefore it should be noted that it may be speculated that the reason why the increase in ratio open arm entries at peak level of the drug was not statistically significant may be the presence of a few outliers.

As mentioned in section 6.3.1.1, a study conducted by Braun *et al.* (1990) found alprazolam to decrease overall anxiety ratings in rats, although the study didn't find a reduction in core PTSD symptoms in reaction to the alprazolam treatment. In the study alprazolam (3.75 mg) was compared to placebo in a 5-week cross-over design, with a two week washout between drug and placebo phases. This is encouraging, not only because it supports the finding in the current study, but because it shows that chronic treatment might be more effective in treating anxiety

than an acute dose. This is in agreement with the clinical situation where treatment of PTSD is usually of a chronic nature. The current study also indicated a possible level of sustainability of the anxiolytic effect of diazepam after chronic administration which may enhance the effectivity of chronic treatment.

It should also be noted that in an article by Vaiva *et al.* (2003), it was speculated that patients with an initial low GABA level could develop PTSD. This study showed that drugs which increase GABA could lower anxiety and have a possibly beneficial effect on PTSD. This further supports a role for diazepam in the treatment of PTSD, as diazepam is a GABA potentiator.

As depicted in **Figure 5-16**, diazepam 3 mg/kg had a significant lowering effect on locomotion at both peak (p=0.0480) and trough (p=0.0064) levels of the drug after chronic administration. As already described in 6.3.1.1, this is not necessarily an indication of diazepam not exerting an anxiolytic effect. As noted previously in this section, in the current study, diazepam 3 mg/kg administered chronically was found to statistically significantly lower aversive behaviour in rats at trough level of the drug, with a strong trend towards decreasing aversive behaviour at peak level of the drug. It is then interesting to note that the current study's finding of a decrease in locomotion in response to chronic treatment with diazepam 3 mg/kg was also noted at both peak and trough concentration of the drug.

6.3.2.2. Effect of lamotrigine 10 mg/kg:

Chronic treatment with lamotrigine 10 mg/kg resulted in an increase in the ratio time spent in the open arms (p=0.0262) in rodents at peak level of the drug, while only a trend towards an increase was evident at trough level, as depicted in **Figure 5-18**.

As mentioned in section 6.2.2, lamotrigine has a long half-life which would mean drug concentrations would stay relatively high for a considerable period of time. Considering that steady state drug levels would have been reached after chronic treatment, this could explain the effect of the drug at both peak and trough levels.

The finding of lamotrigine chronic treatment resulting in less aversive behaviour in rats at peak level of the drug is in accordance with the following:

Clinical studies have suggested the utility of lamotrigine for other intermittent aspects of PTSD, such as flashbacks and nightmares (Hertzberg *et al.*, 1999). Indeed, this study by Herztberg *et*

al. (1999) indicated lamotrigine to be an effective form of treatment in PTSD. In this study, treatment of the patients participating in the study was as follows: 25 mg/day for 2 weeks, 50 mg/day for 2 weeks and 100 mg/day in divided dosages for 1 week. Dosages were then increased by 100 mg every 1 to 2 weeks until achieving a maximal response, reaching a maximum dosage of 500 mg/day or upon developing dose-limiting side effects. The result from this study therefore supports the result from the current study, highlighting the anxiolytic effect of lamotrigine 10 mg/kg after chronic administration. This finding of a positive anxiolytic effect in response to chronic treatment would not be problematic, since most treatment regimes for a disorder such as PTSD would be chronic in nature. It should also be noted that lamotrigine has been acknowledged as augmentation therapy for PTSD by Wells (2003), which also supports the notion of lamotrigine as treatment option for PTSD with a starting dose of 25 mg four times a day and a dosage range of 50 – 500 mg/day.

Lamotrigine, primarily indicated as an anticonvulsant, exhibits anti-kindling properties. Drugs possessing these two distinctive qualities have been considered potential treatments for PTSD (Albucher & Liberzon, 2002). This consideration came in the light of the development of the kindling model as a possible pathophysiological abnormality underlying mood oscillations, moreover, anticonvulsants were found to have anti-kindling properties, offering a possible explanation for their pharmacological effects (Weiss & Post, 1998). These notions are all supported by the evident link between kindling and PTSD and/or anxiety. In PTSD a component of stressor sensitisation is evident, as individuals who have experienced prior traumatic life events appear more likely to suffer from PTSD in reaction to repeated exposure to trauma (Yehuda, 1995c). Moreover, there may be a kindling progression in the emergence of flashbacks, which initially may be triggered by cues linked to the original event and then begin to occur more spontaneously (Post & Weiss, 1998).

Locomotor activity was also assessed in this part of the study and the results are depicted in **Figure 5-19.** It was found that there was a decrease in locomotion at both peak ($p=0.0480$) and trough ($p=0.0064$) level of the drug. The change in locomotion again occurred at the same drug concentrations that caused the decrease in aversive behaviour and in this case it could again be chronic drug treatment leading to higher more constant drug levels that precipitated the effect of the drug on locomotor activity.

It is interesting to note that nonepileptic seizures are associated with an increased prevalence of psychological trauma and PTSD. Bowman (1993) found that 40 % of the nonepileptic seizure sample ($n=27$) qualified for a diagnosis of lifetime PTSD, while 88 % sustained a severe trauma. Other studies demonstrated higher prevalences of both lifetime and current PTSD in

nonepileptic seizure patients compared with epilepsy patients (Arnold & Privitera, 1996). The proposed connection between seizures and PTSD is relevant in the discussion of lamotrigine as possible treatment for PTSD. Because lamotrigine is primarily known for its anticonvulsant properties, this rises the question of whether the capability of lamotrigine as a treatment for PTSD may be due to its anticonvulsant properties after all; curbing the nonepileptic seizures that may underlie PTSD.

Since lamotrigine (and other mood stabilizers such as carbamazepine and gabapentin) has been acknowledged as augmentation therapy for PTSD by Wells (2003), studies of some of the most promising anticonvulsants either alone or as adjuncts to the SSRIs would be of considerable importance in targeting some of the treatment-resistant components of chronic PTSD. Such studies could address the comorbidities that often accompany chronic PTSD, including alcohol and substance abuse, aggression and impulse control disorders and a range of affective and anxiety syndromes (Post, 2004).

Chapter 7: Conclusion

PTSD is an anxiety disorder of which numerous aspects are still insufficiently understood. TDS, an animal model of PTSD, was used to investigate the effects of this procedure on GABA levels in two distinct areas of the brain involved in the neurobiology of PTSD (the hippocampus and frontal cortex). To ascertain whether a relationship exists between the pharmacokinetics of two putative treatment options in PTSD (diazepam and lamotrigine), these drugs' effects on the EPM was investigated at peak and trough levels of the drugs.

This study provides conclusive evidence that:

- GABA levels are changed in response to stress, but not necessarily to a similar extent in all areas of the brain. This is illustrated by the finding in the current study of GABA in the hippocampus remaining unchanged in response to TDS stress, but GABA in the frontal cortex is increased in response to TDS stress. Furthermore, it can be concluded that the change stress causes in GABA levels is probably a lasting effect as the increase in GABA levels in the frontal cortex was found at one day post re-stress with a strong trend towards an increase at seven days post re-stress.

- The peak drug concentration for diazepam occurs at 1 hour after drug administration and for lamotrigine at 2 hours. Furthermore the current study indicated a significant difference in effect between peak and trough levels of diazepam 3 mg/kg after acute administration, as this difference was not found after chronic administration it can be concluded that steady state drug levels were not reached after acute drug administration.

- Chronic drug administration is more advantageous than acute drug administration as its effect is more pronounced. In the current study this was observed with both diazepam and lamotrigine. Diazepam exhibited an anxiolytic effect at trough level of the drug after chronic administration in contrast to an anxiolytic effect only being observed at peak level after acute administration. Lamotrigine exhibited no anxiolytic effect after an acute dose but did indicate anxiolytic properties after chronic administration. In both instances this may be attributed to steady state drug levels only being reached after chronic administration with a more pronounced drug effect at these drug levels. It can therefore also be concluded that in this regard, a pharmacokinetic-pharmacodynamic relationship exists.

7.1 Prospectus:

The current study did provide new information on PTSD and the effects of stress; however, it did leave some looming questions...

- ❖ This study has highlighted the need for further investigation of GABA's role in PTSD and especially the influence stress exerts on GABA concentrations as well as the duration of this influence.

- ● The pharmacokinetic profiles would be even more accurately determined if all the blood samples could be collected from only one test subject, as inter-subject variation does play a role. Due to the fact that a rat could only provide 1 blood sample of 0.5 ml, it would seem that human subjects would have to be used in order to achieve the ideal conditions for this experiment. To investigate the pharmacokinetic-dynamic relationship further it will be necessary to obtain blood levels and effect measurements at more time points in one dosing interval.

- ❖ Lamotrigine should be further investigated with regard to its anxiolytic effects, all the encouraging theory supporting this notion and the one finding from this study certainly calls for this.

It is the hope of the conductors of the current study that the results from this study and possible subsequent studies would contribute to expanding the growing knowledge of PTSD and especially the treatment of PTSD.

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