Extended characterisation and validation of an animal model of post-traumatic stress disorder: Behavioural, molecular and pharmacological studies

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Abstract

Posttraumatic Stress Disorder (PTSD) is an anxiety disorder precipitated by exposure to a severe traumatic event. Given the socio-economic impact of the disorder, and the increasing rates of trauma worldwide, PTSD is set to become a major global health problem. There exists a clear need for the development of drug treatments specifically for PTSD, yet the neurobiology of the disorder remains to be completely elucidated. In this regard, animal models are critical tools in the study of the pathophysiological mechanisms of stress, as well as in the testing of potential drug treatments. These animal models should be well-validated, reliable and generalisable (factors that are often overlooked in validation studies) to ensure that findings from the models will be meaningful and that research animals are not used unnecessarily.

Earlier behavioural, endocrine and pharmacological studies in our laboratory had established that the time-dependent sensitisation (TDS) model (single prolonged stress + re-stress) presents with noteworthy construct-, face- and predictive validity. However, subsequent studies in our laboratory and elsewhere have yielded contrasting or inconclusive results, especially with regard to behavioural changes. The primary aim of the current study was therefore to re-investigate the TDS model as analogous PTSD model, with regard to cognitive performance, anxiety-like behaviour and endocrine function. The original validation was also extended by examining arousal behaviour and the influence of chronic fluoxetine administration on TDS-induced endocrine changes. Furthermore, the robustness of the model was investigated by subjecting it to more stringent testing, using a greater range of parameters and criteria provided by computerised behavioural monitoring with powerful software. The reliability and generalisability of the TDS model was also studied by comparing results obtained from the current study with those from the original validation study. Finally, with the increasing importance of neuronal plasticity and resilience in stress-related disorders and antidepressant action, the effects of TDS stress on a broad range of cellular plasticity and resilience proteins was studied in selected limbic brain regions.

Sprague-Dawley and Wistar rats were left undisturbed (controls) or subjected to the TDS model consisting of a single prolonged stress (SPS) (2 hours restraint, 15 minutes forced swim, halothane exposure) and a re-stress (RS) (20 minutes forced swim) 7 days later.
Seven days after the re-stress, animals were tested for spatial learning and memory, anxiety-like behaviour or arousal in the Morris Water maze (MWM), elevated plus maze (EPM) or acoustic startle response (ASR), respectively. The activity of endocrine function as measured by plasma corticosterone was also investigated in control and TDS behavioural test exposed (Sprague-Dawley and Wistar), test naive (Sprague-Dawley and Wistar) and test naive saline or fluoxetine treated (Wistar) rats. Finally, the expression of selected cellular plasticity and resilience proteins was determined by Western blot in the hippocampus and frontal cortex of test naive Wistar rats.

In contrast to the findings of the original validation studies, TDS stress failed to have a marked effect on spatial learning and memory and anxiety-like behaviour, suggesting a lack of reliability and generalisability of the TDS model. In the extended characterisation of the model, TDS stress also did not induce any significant changes in arousal. Data from the behavioural studies indicate a lack of robustness of the TDS model, which may be due to habituation to the re-stress procedure. TDS stress was, however, able to significantly (bidirectionally) alter endocrine function, while TDS stress induced suppression of corticosterone was prevented by chronic fluoxetine administration. These data suggest face validity, as well as possible construct- and predictive validity for the TDS model with regard to endocrine function. Finally, while fluoxetine had notable effects on the expression, phosphorylation and/or relative activation of cellular plasticity and resilience proteins tested, TDS stress failed to have a marked effect on these same proteins. However, the latter negative findings may not necessarily be an indication of a lack in validity or robustness of the TDS model.

Although the TDS model demonstrated face validity, as well as possible construct- and predictive validity in terms of endocrine function, data from the behavioural studies suggest that the model lacks reliability and generalisibility and hence, relevance. The current data suggest that improvements to the model include omission of the re-stress procedure, or alternatively, replacement of the re-stress with a situational reminder of the SPS. The effects of stress on cellular plasticity and resilience proteins warrant further investigation with such an improved animal model. In conclusion, the current study therefore serves to highlight the importance of thorough validation of any behavioural animal model, especially confirmation by investigators other than those involved in the original studies.
**Opsomming**

Posttraumatiese stres sindroom (PTSS) is 'n angsteurnis wat veroorsaak word na blootstelling aan 'n ernstige, tramatiese gebeurtenis. As gevolg van die sosio-ekonomiese impak van hierdie sindroom en die stygende insidensie van trauma wêreldwyd, kan PTSS in 'n globale gesondheidsprobleem ontaard. Daar bestaan 'n duidelike behoefte aan die ontwikkeling van geneesmiddels spesifiek vir die behandeling van PTSS, maar die neurobiologie van die sindroom moet steeds ten volle verklaar word. In hierdie verband is diermodelle krities vir die bestudering van patofisiologiese meganismes van stres, sowel as vir die toets van moontlike geneesmiddel behandeling. 'n Diermodel behoort goed gevalideer, betroubaar en veralgemeenbaar (faktore wat gereeld mis gekyk word in geldigheid studies) te wees, om te verseker dat bevindings van 'n studie betekenisvol en relevant sal wees en dat proefdiere nie onnodig gebruik word nie.

Vorige gedrags-, endokriene- en farmakologiese studies in ons laboratorium het vasgestel dat die tydsafhanklike sensitisering (TAS) model (enkele verlengde stres + herstres) noemenswaardige konstrukt-, fenomenologiese- en voorspelbaarheid geldigheid besit. Daaropvolgende studies in ons laboratorium en elders het egter kontrasterende of onsekere resultate opgelewer. Die primêre doelwit van die huidige studie was dus om die TAS model as 'n analoog model van PTSS te herondersoek in terme van geheue, angstigheid en endokriene funksie. Die oorspronklike geldigheid is ook uitgebrei deur opwekkings gedrag (eng: arousal) en die invloed van kroniese fluoksetien toediening op TAS geïnduseerde endokriene verandering te ondersoek. Verder is die robuustheid van die model ook onderzoek deur dit te onderwerp aan 'n wyer reeks parameters en kriteria moontlik gemaak deur gerekeneriseerde gedragsmonitoring met krachtige sagteware. Die betroubaarheid en veralgemenning van die model is ook bestudeer, deur die resultate van die huidige studie te vergelyk met resultate van die oorspronklike geldigheid studie. Ten slotte, met die toenemende belangrikheid van neuronale plastisiteit en elastisiteit in stres verwante siekte en die aksie van antidepressante, is die effekte van TAS stres bestudeer op 'n wye reeks sellulêre plastisiteit- en elastisiteit proteïne.

Sprague-Dawley en Wistar rotte was onverstoord gelaat (kontrole) of onderwerp aan die TAS model wat bestaan uit 'n enkele verlengde stress (SPS) (2 ure immobolisasie, 15 minute geforseerde swem, halotaan blootstelling) en 'n herstres (RS) 7 dae later. Sewe
dae na die herstres is diere getoets vir ruimtelike leer- en geheue vermoë, angstigheid en opgewektheid in die "Morris water maze" (MWM), "elevated plus maze" (EPM) en die akoestiese skrik respons (ASR), onderskeidelik. Die aktiwiteit van endokriene funksie, soos gemeet deur plasma kortikosteroon is ook ondersoek in kontrole en TAS gedrag toets blootgestelde- (Sprague-Dawley en Wistar), toets naïef- (Sprague-Dawley en Wistar) en toets naïef saline of fluoksetien behandelde (Wistar) rotte. Ten slotte is die uitdrukking van geselekteerde sellulære plastisiteit- en elastisiteit proteïene ook bepaal deur Western blot analysie in die hippocampus en frontale korteks van toets naïef, Wistar rotte.

In teenstelling met die bevindings van die oorspronklike studies het TAS stres nie 'n beduidende effek op ruimtelike leer- en geheue vermoë en angstigheid gehad nie, wat 'n aanduiding is van 'n gebrek aan die model se betroubaarheid en veralgemening. In die uitgebreide karakterisering van die model het TAS stres ook nie enige betekenisvolle veranderinge in opgewektheid geïnduseer nie. Data van die gedragstudies dui op 'n tekort aan robuustheid van die TAS model, wat moontlik as gevolg van akklimatisering aan die herstres prosedure is. In teenstelling, TAS stres was in staat om endokriene funksie beduidend (tweerigtingsgewys) te verander, terwyl TAS stres geïnduseerde onderdrukking van kortikosteroon voorkom is deur kroniese fluoksetien toediening. Hierdie data dui fenomenologiese-, sowel as moontlike konstruk- en voorspellings geldigheid vir die TAS model aan, in terme van endokriene funksie. Ten slotte, terwyl fluoksetien beduidende effekte op die uitdrukking, fosforilasie en/of relatiewe aktivering van sellulære plastisiteit- en elastisiteit proteïene gehad het, het TAS nie daarin geslaag om 'n beduidende effek op dieselfde proteïene te hê nie. Nieteenstaande, laasgenoemde negatiewe bevindinge dui nie noodwendig op 'n tekort aan geldigheid of robuustheid van die TAS model nie.

Alhoewel die TAS model fenomenologiese-, sowel as moontlike konstruk- en voorspellingsgeldigheid het in terme van endokriene funksie, dui data van die gedragstudies daarop dat die model 'n gebrek aan betroubaarheid en veralgemeenbaarheid, en gevolglik, relevansie het. Die huidige data stel voor dat verbeterings aan die model weglating van die herstres, of as alternatief, vervanging van die herstres met 'n situasie-herlewing, insluit. Die effekte van stres op sellulære plastisiteit- en elastisiteit proteïene regverdig verdere ondersoek met so 'n verbeterde
model. Ter afsluiting, die huidige studie benadruk die belangrikheid van deeglike validering van enige gedragsmodel, veral bevestiging deur ondersoekers wat nie betrokke was by die oorspronklike studies nie.
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1.1 Problem Statement

Post-traumatic Stress Disorder (PTSD) is an anxiety disorder precipitated by exposure to a severe traumatic event (Rauch and Foa, 2003). Despite considerable effort, the neurobiology of PTSD remains to be completely elucidated and pharmacological treatment of the disorder is based on drugs developed for other disorders, which attempt to relieve symptoms rather than cure (Turnbull, 1998b). In addition, most of these drug treatments have been suggested to have limited efficacy (Freeman, 2006) and a delayed onset of action (Gelenberg and Chesen, 2000; Regen and Anghelescu, 2006).

PTSD is often treatment resistant (Hamner et al., 2004; Harvey, 2006) and is characterised by high comorbidity (Rauch and Foa, 2003), high morbidity, diminished quality of life (Boscarino, 2006) and a substantial socio-economic impact (Rauch and Foa, 2003; Vieweg et al., 2006). Given these factors and the increasing rates of trauma worldwide, PTSD is set to become a major global health problem (Connor and Butterfield, 2003). It is clear that there exists an urgent need for the improvement of current drug therapies, or for the development of new treatment strategies. In this regard, animal models are critical to study the cause of stress-induced changes, investigate the underlying physiological and neuronal mechanisms, and test potential drug treatments for their efficacy and safety (Stam, 2007a). However, to be relevant, an animal model should be well-validated, reliable, and generalisable (Van der Staay, 2006). Only animal models fulfilling these criteria should continue to be used, to ensure that the findings will be meaningful (Bird and Parlee, 2000). Importantly, negative findings from animal model studies should also be considered to ensure that invalid models are abandoned and the unnecessary use of animals is reduced (Van der Staay, 2006).
Earlier studies in our laboratory had evaluated the effects of time-dependent sensitisation (TDS) stress (single prolonged stress + re-stress) using the Morris water maze (MWM), elevated plus maze (EPM) and endocrine- and pharmacological studies (Harvey et al., 2003; Harvey et al., 2004a; Naciti, 2002). These studies established that the TDS model presents with noteworthy construct-, face- and predictive validity, as PTSD often presents with impaired working memory (Praag, 2004), anxiety (Damsa et al., 2005) and low basal corticosterone (Yehuda, 2006), and SSRI treatment has been shown to have some efficacy in the treatment of the disorder (Asnis et al., 2004). However, subsequent studies with the TDS model in our laboratory have found contrasting results with regard to endocrine function and inconclusive results with regard to anxiety-like behaviour in the EPM (Jeeva, 2004). Furthermore, studies in another laboratory have found that although a similar TDS procedure was able to induce changes in endocrine function and hippocampal neurotrophin levels, no differences could be detected in anxiety-like behaviour in the EPM and open field (Uys et al., 2006a).

1.2 Study Aims

Since completion of the initial validation study, a new behavioural laboratory has been set up, including new digital behavioural monitors with powerful analysis software. Given the abovementioned conflicting results, in order to establish replicability or reliability, as well as the robustness of the TDS model as an analogous PTSD model, it was now reinvestigated and subjected to more stringent testing in the MWM and EPM using a greater range of parameters and criteria provided by Accutrac Software® digital analysis. Moreover, the behavioural evaluation was extended by assessing arousal as measured in the acoustic startle response (ASR) using an automated startle system. In both the EPM and ASR, individual susceptibility to TDS stress, as measured by behavioural cut-off criteria (Cohen and Zohar, 2004; Cohen et al., 2004 & 2006), was studied in Sprague-Dawley and Wistar rats, two rat strains that are known to have different sensitivities to stress (Bekris et al., 2005; Staples and McGregor, 2006). These analyses were performed taking into account also the fact that the known incidence of PTSD is only 9-30% of an exposed population (Breslau et al., 1998; Kessler et al., 1995).
Following these extended behavioural tests, the effects of TDS stress on endocrine function and a broad range of signalling proteins was studied in selected limbic brain regions known to be influenced by stress as well as drug treatment. The choice of signalling protein was based on the neuronal plasticity hypothesis of the actions of antidepressants (Duman et al., 1999; Gould and Manji, 2002; Picchini et al., 2004), which are extensively used in the treatment of PTSD (Asnis et al., 2004) and that PTSD and other stress-related disorders are causally linked to altered activity and function in certain cellular resilience pathways (Charney et al., 2004; Manji and Duman, 2001). In these studies, the effect of chronic fluoxetine treatment was studied and used to confirm in the Western blot protocol the rationale to access the above proteins following TDS stress. Additionally, predictive validity testing was extended by investigating the influence of the selective serotonin re-uptake inhibitor (SSRI) fluoxetine on TDS-induced neuroendocrine changes.

1.2.1 Primary Aim: Re-Investigation of the TDS Model

The primary aim of the study was to re-investigate TDS stress as an animal model of PTSD, in terms of face, construct and predictive validity, as well as reliability, generalisability and relevance. This was accomplished by specific secondary aims that included:

- Investigation of the effect of TDS stress on cognitive ability (spatial memory acquisition and consolidation) by using a validated animal test, the MWM (face validity).
- Investigation of the effect of TDS stress on anxiety-like aversive behaviour by using a well-established, validated animal test, the EPM (face validity).
- Investigation of the effect of TDS stress on the magnitude, habituation and pre-pulse inhibition of the acoustic startle response, using a validated protocol (face validity).
- Investigation of individual susceptibility to stress, by analysing EPM and ASR data using modified versions of previously published cut-off criteria for extreme behavioural response.
• Investigation of the effect of TDS stress on hypothalamic-pituitary-adrenal (HPA) axis activity, as measured by plasma corticosterone (face and construct validity).

• Investigation of the effect of chronic fluoxetine administration on HPA axis activity, as measured by plasma corticosterone (predictive validity).

• Investigation of the effects of TDS stress on the hippocampal and frontal cortex expression of selected signalling proteins involved in neuronal plasticity, resilience and/or survival (construct validity).

• Investigation of the reliability and generalisability of the TDS model (construct validity and relevance) by comparing data from the current study to previous validation studies in our laboratory.

1.2.2 Secondary Aim: Validation of Protocols

1.2.2.1 Behavioural Tests

Because the reliability of an animal model depends on the sensitivity and adequacy of the test used to measure the dependent variable (behaviour) (Van der Staay, 2006), the behavioural test protocols used to assess the effects of the TDS model were extensively validated. Thus, specific aims of this phase of the study were to:

• Pharmacologically validate the MWM test protocol as a measure of working memory function, using a digital tracking system.

• Pharmacologically validate the EPM test protocol, as a measure of anxiety, using a digital tracking system.

• Parametrically and pharmacologically validate the ASR test protocol, as a measure of hyperarousal, using an automated startle system.

1.2.2.2 Western Blotting

This phase of the study aimed to set up Western blotting in the laboratory. Specific aims were as follows:
Standardisation/optimisation of Western blotting conditions for each antibody to be used in the TDS studies.

Validation of the densitometric analysis method using the ChemiDoc XRS system with Quantity One® 1-D analysis software (Bio-Rad).

Confirming that the standardised Western blotting conditions and method of densitometric analysis are capable of detecting changes in the expression of proteins induced by an external challenge. In this case, pharmacological challenge with the antidepressant, fluoxetine, was studied. These studies would also serve to reaffirm the range of proteins to be measured following TDS stress.

1.3 Project Layout

1.3.1 Behavioural Validation Studies

The behavioural test protocols of the MWM and EPM were validated in Sprague-Dawley rats with drugs known to disrupt spatial learning and memory (scopolamine), or induce anxiety (mCPP and β-carboline), respectively (Janas et al., 2005; Pähkla et al., 2000; Wallis and Lal, 1998). The protocol for the ASR was designed based on the results of the parametric validation study with both Sprague-Dawley and Wistar rats, and validated pharmacologically in Sprague-Dawley rats with d-amphetamine, a drug known to potentiate the ASR and disrupt pre-pulse inhibition (Bell et al., 2003).

1.3.2 Western Blot Set-Up Studies

Western blotting conditions were standardised for each antibody to be used. Thereafter, the method of densitometric analysis was validated by constructing a protein concentration curve. Finally, the ability of the standardised conditions and validated densitometric analysis to detect changes in the expression of selected proteins following chronic fluoxetine challenge in Wistar rats was determined.
1.3.2 TDS Stress Studies

Sprague-Dawley rats were exposed to TDS stress or left undisturbed to serve as controls. TDS consisted of an initial single prolonged stress (SPS) (2hrs restraint, 15 minutes forced swim, halothane exposure), followed by a re-stress (RS) (20 minutes forced swim) 7 days later. Rats were tested for behavioural stress sensitisation 7 days after the re-stress. The effect of TDS stress on HPA axis activity in rats exposed to behavioural tests, as well as test naive rats, was also investigated. The behavioural and endocrine studies were repeated in Wistar rats, a strain known to be more stress sensitive and anxiety prone than Sprague-Dawleys (Bekris et al., 2005; Rex et al., 2004; Staples and McGregor, 2006).

The effect of chronic fluoxetine on TDS stress induced alterations in HPA axis activity in Wistars was then studied. Thereafter, and again using Wistar rats, the final aspect of the study accessed the influence of TDS stress on the hippocampal and frontal cortex expression of selected proteins involved in neuronal plasticity, resilience and survival.
2.1 Introduction

As mentioned in chapter 1, the aim of the study was to re-investigate the relevance of the TDS model as a model of post-traumatic stress disorder (PTSD) in terms of face, construct and predictive validity. Because a certain amount of clinical knowledge is required for the assessment of animal models, the current chapter will provide an overview of the literature with regard to symptoms, epidemiology, response to treatment and neurobiology of PTSD.

2.2 Symptoms

PTSD is an anxiety disorder of characteristic symptoms following exposure to an extreme traumatic stressor (Rauch and Foa, 2003) involving death, injury or a threat to the physical integrity of self or another person. Examples of traumatic events include military combat, violent personal assault (rape, robbery, mugging, physical attack), being kidnapped, being taken hostage, incarceration as prisoner of war, natural or manmade disasters, severe automobile accidents, or being diagnosed with a life-threatening illness (APA: DSM-IV-TR, 2002).

The symptoms of PTSD can be divided into three clusters, namely re-experiencing, avoidance and/or numbing and hypersrosual and/or hypervigilance (APA: DSM-IV-TR, 2002). Re-experiencing symptoms involve the reliving of the trauma, commonly in the form of recurrent and distressing flashbacks. These recollections may induce a dissociative state and induce psychological and/or physiological stress reactions such as panic-attacks (APA: DSM-IV-TR, 2002). The second symptom cluster,
avoidance/numbing, involves the avoidance of thoughts, feelings, conversations, activities, places, or people associated with the trauma. Amnesia of aspects of the trauma, anhedonia, detachment or estrangement from others, blunted emotions and a sense of a foreshortened future are also common in PTSD (APA: DSM-IV-TR, 2002). Finally, symptoms in the third cluster, hyperarousal/hypervigilance, include sleep disturbances, irritability, anger, impaired concentration, hypervigilance and exaggerated startle response (APA: DSM-IV-TR, 2002).

To establish a diagnosis of PTSD, the person's response to the traumatic event must involve intense fear, helplessness or horror and at least one symptom of re-experiencing, three symptoms of avoidance/numbing and two symptoms of hyperarousal and/or hypervigilance must be present for one month or more. In addition, these disturbances must cause clinically significant distress and/or impairment in social, work-related or other important areas of functioning in the patient (APA: DSM-IV-TR, 2002).

2.3 Epidemiology

Despite the fact that the development of PTSD can be traced back to a severe traumatic event, the reason why some people exposed to such as trauma go on to develop the disorder and others do not remains unknown. Studies of PTSD have investigated several epidemiological factors of the disorder that may provide clues to understanding this susceptibility to stress and stress-related disorders. For the purpose of this study, the epidemiology of PTSD will be discussed with regard to its prevalence, comorbidity, risk-factors and clinical course.

The two major epidemiological PTSD studies (1990-1992 National Comorbidity Survey (NCS), 1996 Detroit Area Survey of Trauma) that have been conducted since its recognition as a distinct anxiety disorder have both been performed on Americans. It should be kept in mind, however, that data form these studies cannot be applied directly to other countries because of cultural differences. It is likely that large portions of populations in developing nations have been exposed to traumatic events such as terrorism, forced relocation and violent crime. This raises the possibility that the overall exposure to traumatic events and the prevalence of PTSD worldwide may be considerably
higher than that in the USA (Galea et al., 2005; Schnurr and Friedman, 1997). Nevertheless, because comparable international data are limited, studies like the NCS and Detroit Area Survey of Trauma provide useful insight into the epidemiology of PTSD.

### 2.3.1 Prevalence

The NCS and Detroit Area Survey of Trauma reported a lifetime prevalence of 7.8% and 9.2% by using DSM-III-R and DSM-IV criteria, respectively (Breslau et al., 1998; Kessler, 1995). The NCS also found that after 6 years, about one third of PTSD patients did not remit regardless of treatment and concluded that PTSD is a highly prevalent lifetime disorder that often persists for years (Kessler, 1995).

Although there have been relatively few epidemiological studies of trauma disorders in South Africa, research suggests that South Africans may be suffering from PTSD in numbers far greater than the average. In this regard, one local epidemiological study has reported a PTSD prevalence of 19% (Suliman et al., 2005). Another South African based study found that of the 23% exposed to one or more violent events, 78% presented with symptoms of PTSD (Hirschowitz & Orkin, 1997). Statistics support that South Africa is an extremely violent country (Hamber and Lewis, 1997; van Dijk, 1996), a factor that may contribute to the greater trauma exposure and subsequent development of PTSD.

### 2.3.2 Comorbidity

Epidemiological studies indicate that PTSD has a high comorbidity with other anxiety disorders, major depressive disorder, alcohol abuse and substance abuse (Kessler, 1995). It has also been suggested that psychotic symptoms may occur in 30-40% of PTSD sufferers and may even approach the severity observed in schizophrenia (Connor and Butterfield, 2003; Hamner et al., 2000). Finally, PTSD may also be comorbid with other non-psychiatric medical conditions such as cardiovascular disease, diabetes, gastrointestinal disease and endocrinological disorders (Boscarino, 1997; Connor and Butterfield, 2003).
Although PTSD often precedes other comorbid diagnoses, it usually succeeds at least one diagnosis, suggesting that a prior history of mental disorder may be a risk factor for the development of PTSD (Kessler et al., 1995). Pre-existing depression, for example, may render individuals more vulnerable to developing PTSD in the aftermath of a traumatic experience. Conversely, the presence of PTSD may increase the risk for first onset of major depressive disorder (Breslau et al., 1997; Kessler et al., 1995). These data suggest the possibility of a shared vulnerability for PTSD and major depression, which is supported by the finding that the disorders share many other risk factors such as female gender, childhood adversity and a psychiatric family history (Brewin et al., 2000; Burt and Stein, 2002; Gilman et al., 2003). Taken together, these findings suggest that comorbid PTSD and major depression after exposure to a traumatic event may represent a single traumatic stress disorder. In contrast, other researchers maintain the view that PTSD and major depression in the aftermath of trauma are two distinctly separate disorders (O’Donnel et al., 2004).

### 2.3.3 Risk Factors

Most adults will not experience a major mental health disorder as a consequence of exposure to a traumatic event or subsequent ongoing stressors (Turnbull, 1998a). Instead, a large proportion of the population will have a brief acute stress response to a trauma that will stabilise over time. However, the remainder of the population will experience an ongoing stress response, eventually leading to the development of PTSD (Davidson et al., 2004). The basis of why some people show maladaptive responses upon exposure of trauma and go on to develop PTSD while others do not is unknown. Responses to stress show striking individual variation and individual vulnerabilities could, in principle, exert their influence via pre-existing psychobiological characteristics, perception and response during the trauma, or post-traumatic coping strategies and social support (Charney et al., 2004; De Kloet et al., 2005; Ozer et al., 2003).

In a meta-analysis of risk factors for PTSD in trauma-exposed adults, three categories of risk factors emerged. The first group of factors predicted PTSD in some populations but not in others (gender, age at trauma, race). The second group of factors predicted PTSD more consistently, but to a varying extent according to the populations studied and the
methods used (education, previous trauma, general childhood adversity). Finally, a third group of factors had more uniform predictive effects (psychiatric history, reported childhood abuse, family psychiatric history) (Brewin et al., 2000).

### 2.3.4 Clinical Course

The DSM-IV-TR specifies that PTSD can be acute, chronic or delayed. In acute PTSD, the duration of symptoms is less than 3 months, whereas a chronic case of the disorder is one in which symptoms last 3 months or longer. Delayed onset of PTSD is diagnosed when at least 6 months have elapsed between the traumatic exposure and the onset of symptoms (APA: DSM-IV-TR, 2002). Most cases of PTSD are not acute, with an estimated 90% and 70% of PTSD cases lasting longer than three months or one year, respectively. Strikingly, more that one third of PTSD patients may never recover (Kessler et al., 1995). Although onset of PTSD symptoms may be delayed for months or even years, a delayed onset is not the norm. A stable course is also not the norm, since individuals who have been asymptomatic for years may experience recurrence of PTSD symptoms (Kessler et al., 1995; Schnurr et al., 2002).

### 2.4 Treatment

PTSD is very complex in its epidemiology, comorbidity, neurobiology and symptomology. As a result, it has been difficult to develop effective treatment strategies for the disorder, yet appropriate treatment is essential to improve the quality of life of sufferers (Davidson et al., 2004).

General treatment goals for PTSD include reduction of symptom severity, the prevention or reduction of comorbid conditions, improvement of functioning in daily life, and prevention of relapse (APA: Practice Guidelines, 2004). The selection of specific treatment strategies depends on many factors, including the patient’s age, most prominent symptoms, comorbid psychiatric and general medical conditions and stressor type. The safety, effectiveness, acceptability and time to onset of action of the treatment should also be taken into account (Foa et al., 1999). Treatment options include psychotherapy, education and support, and pharmacotherapy. Although psychotherapy, education and
support can be very effective in alleviating PTSD symptoms (APA: Practice Guidelines, 2004; Bradley et al., 2005), for the purpose of the current study only pharmacotherapy will be discussed further.

The principle goals of pharmacotherapy in PTSD are to reduce PTSD symptoms, improve resilience to stress, reduce comorbidity and disability, and improve quality of life (Davidson et al., 2004). The current approach to the pharmacological treatment of PTSD is empirical rather than theoretical (Friedman, 2000). Most drugs tested in PTSD were developed as antidepressants and only later shown to have efficacy in anxiety disorders. Given the high comorbidity and symptom overlap between PTSD and major depressive disorder, it is reasonable to expect antidepressants to demonstrate some efficacy in PTSD. However, despite the clinical similarities between the two disorders, PTSD is more complex, with a different underlying pathophysiology. Thus, it is argued that new drugs should be developed and tested specifically for PTSD, rather than just using those developed for other disorders (Friedman, 1997).

### 2.4.1 Empirical Treatment Options

With regard to the empirical approach, nine classes of drugs have demonstrated some efficacy in the treatment of PTSD symptoms, namely selective serotonin re-uptake inhibitors (SSRIs), novel antidepressants, tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), benzodiazepines, non-benzodiazepine anxiolytics and hypnotics, anticonvulsants, mood stabilisers and antipsychotics.

#### 2.4.1.1 SSRIs

As their name suggests, the SSRIs selectively block the reuptake of synaptic serotonin (5-HT) by allosterically modulating the 5-HT transporter (Kent et al., 1998; Vaswani et al., 2003). Their clinical efficacy however, may not be due to this straightforward increase in extracellular 5-HT. Autoreceptors located on the presynaptic cell body (5-HT1A) and axon (5-HT1D), regulate the net amount of 5-HT available in the synapse. It is hypothesised that long-term SSRI treatment desensitises these receptors, thereby increasing the availability of synaptic 5-HT (Kent et al., 1998).
SSRIs are usually well tolerated and have a benign side effect profile. Gastrointestinal complications and sexual dysfunctions are the most common side effects and tolerance to these side effect may develop with long-term treatment (Albucher and Liberzon, 2002; Vaswani et al., 2003).

Evidence from several case reports and open-label studies suggested SSRIs to be beneficial in the treatment of PTSD (for example Brady et al., 1995; Folnegovic-Smalc et al., 1997; Frommberger et al., 2004; Marshall et al., 1998; Nagy et al., 1993; Rothbaum et al., 1996). These initial findings of SSRI efficacy in PTSD have since been confirmed in several large, randomised, double-blind controlled trials of fluoxetine, sertraline, paroxetine and fluvoxamine (for example Brady et al., 2000; Connor et al., 1999; Davidson et al., 2001; Marshall et al., 2001; Martenyi et al., 2002; Spivak et al., 2006).

In summary, several open trials and published controlled-trials indicate that the SSRIs are effective in improving PTSD symptoms in two or all three of the symptom clusters. In addition to being the most studied and effective drugs for PTSD, the SSRIs have a relatively benign side effect profile, making these agents the first-line pharmacological treatment option for PTSD. Sertraline and paroxetine are currently also the only Food and Drug Administration-approved drugs for PTSD (APA: Practice Guidelines, 2004). If, however, the SSRIs are not tolerated well or are ineffective, other drugs should be considered (Asnis et al., 2004).

### 2.4.1.2 Novel Antidepressants

Trazodone, venlafaxine, mirtazepine and bupropion all potentiate serotonergic neurotransmission through a number of different mechanisms. Although some may block the uptake of 5-HT like the SSRIs, they are none-selective (Asnis et al., 2004). Case reports and open-label trials suggest some benefits for most of the these drugs in the treatment of PTSD symptoms (Connor et al., 1999; Hamner and Frueh, 1998; Hargrave, 1993; Hertzberg et al., 1996; Kim et al., 2005). Double-blind, placebo-controlled studies also support the use of venlafaxine (Davidson et al., 2006) and mirtazapine (Davidson et al., 2003), but not bupropion (Hertzberg et al., 2001) in PTSD. Finally, no double-blind, placebo-controlled studies have been conducted for trazodone in PTSD.
Tianeptine is a clinically effective antidepressant agent with a favourable side effect profile and a novel neurochemical profile. In contrast to the SSRIs and most other antidepressants, tianeptine increases 5-HT uptake in the brain (Wagstaff et al., 2001). With recent studies reporting an ability of tianeptine to reverse the actions of stress and glucocorticoids on dendritic remodelling in animal models of stress (McEwen and Chattarji, 2004) there has been some interest in its role in the pharmacotherapy of PTSD. Indeed, recent studies indicate that the drug is highly effective in treating PTSD (Önder et al., 2006), especially the chronic form of the disorder (Aleksandrovskiï et al., 2005).

### 2.4.1.3 TCAs

The TCAs are catecholamine/indolamine reuptake blockers, with varying degrees of norepinephrine (NE) and 5-HT reuptake inhibition depending on the drug (Asnis et al., 2004). The group also antagonises α2-adrenergic-, muscarinic- and histaminergic receptors, resulting in many of their known side effects including anticholinergic, sedative and cardiac effects (Glod, 1996).

Although not licensed for the treatment of PTSD, TCAs are sometimes considered as an alternative treatment (VA/DoD clinical practice guideline group, 2003). These antidepressants have been in use for much longer than the SSRIs and the trials of TCAs in PTSD are much older. Controlled trials (Davidson et al., 1990; Kosten et al., 1991; Reist et al., 1989) and several case studies and open-label trials (Burnstein, 1984; Falcon et al., 1985; Kauffmann et al., 1987) demonstrate efficacy of imipramine and amitriptyline, but not desipramine in global improvement of PTSD. Despite higher dropout rates due to poor tolerability, TCAs like imipramine and amitriptyline should therefore be considered as valid alternatives in patients who are intolerant to SSRIs (Albucher and Liberzon, 2002). Of note is that in all of the controlled- and most of the uncontrolled trials for TCAs, the participants were combat veterans suffering from chronic PTSD. Their modest efficacy in this notoriously treatment resistant population should therefore be given serious consideration (Albucher and Liberzon, 2002).
2.4.1.4 MAOIs

This mechanism of action of this class of antidepressants involves the inhibition of the enzyme monoamine oxidase (MAO) and subsequently, the potentiation of serotonergic, noradrenergic and dopaminergic neurotransmission (Asnis et al., 2004). The MAOIs also block histaminergic- and α2-adrenergic receptors, which may contribute to their adverse effects (Asnis et al., 2004).

Interest in the possible role of MAOIs in the treatment of PTSD has led to numerous case reports and open-label trials (for example Davidson et al., 1987; Neal et al., 1997). Several controlled studies have also been undertaken, but their results are inconsistent (Baker et al., 1995; Katz et al., 1994; Kosten et al., 1991; Shestazky et al., 1987). Despite these mixed findings, PTSD patients treated with this class of drugs show greater global improvement than those treated with TCAs. Unfortunately, due to patients needing to follow a restrictive diet to prevent hypertensive crisis, dropout rates on MOAIs, especially phenelzine, are high. As with the TCA trials, most MOAI studies were performed in combat veterans known to be treatment-resistant and as a result, the MOAIs should be considered in treatment resistant PTSD (Albucher and Liberzon, 2002; VA/Do clinical practice guideline group, 2003).

2.4.1.5 Benzodiazepines

Benzodiazepines such as alprazolam, clonazepam, lorazepam and temazepam act as agonists at the gamma-aminobutyric acid (GABA)A receptor, thereby potentiating the action of GABA at its receptor (Borchardt, 1999). As a result of their clinical anxiolytic efficacy, as well as their ability to decrease arousal and promote sleep, there has been some interest in their possible use in PTSD. A number of retrospective studies and case reports (Bleich et al., 1986; Feldmann, 1987; Lowenstein et al., 1988) suggested some efficacy for benzodiazepine treatment in PTSD, but subsequent controlled studies failed to confirm the early positive findings (Braun et al., 1990; Shalev et al., 1998; Shalev and Rogel-Fuchs, 1992).
It has been hypothesised that early treatment of trauma survivors with benzodiazepines may offer protection towards future development of PTSD (Gelpin et al., 1996). However, both open-label (Gelpin et al., 1996) and double-blind, placebo-controlled (Mellman et al., 2002) studies have found that the benzodiazepines are ineffective in preventing PTSD and may even increase the risk of developing PTSD and major depression. Their abuse potential, together with the possibility that withdrawal from benzodiazepines may exacerbate PTSD symptoms (Risse et al., 1990) has further discouraged their use in the treatment of PTSD.

2.4.1.6 Non-Benzodiazepine Anxiolytics and Hypnotics

Buspirone is a novel non-benzodiazepine anxiolytic that acts as a partial 5-HT$_{1A}$-receptor agonist (Argyropoulos et al., 2000). In contrast to the benzodiazepines, buspirone demonstrates a low potential for abuse and withdrawal symptoms (Lader, 1987). Data from several open-label trials (Duffy and Malloy, 1994; Fichtner and Crayton, 1994; Wells et al., 1991) indicate that buspirone may be a safe and effective alternative treatment for PTSD, but larger, controlled studies are needed to confirm these preliminary results.

Zolpidem is a nonbenzodiazepine drug which binds with low affinity to $\alpha_5$-containing GABA$_A$-receptor subtypes (Rush, 1998). It has been suggested that the drug may be useful in the treatment of insomnia associated with PTSD, and it may provide advantages over other medications for inducing and maintaining sleep in PTSD (Dieperink and Drogemuller, 1999).

2.4.1.7 Anticonvulsants and Mood Stabilisers

Anticonvulsive drugs have been reported to be effective in bipolar disorder, as well as in reducing aggression in chronic psychiatric patients (Dunn et al., 1998). Kindling and neuronal sensitisation processes have been suggested to contribute to the pathophysiology of PTSD (Post et al., 1995), leading to an interest in anticonvulsants and mood stabilising drugs in the treatment of the disorder (Hageman et al., 2001).
Case reports and open-label studies indicate some efficacy for the anticonvulsants carbamazepine and oxcarbazepine (Ford, 1996; Looff et al., 1995; Malek-Ahmadi and Hanretta, 2004), valproate (Berigan and Holzgang, 1995; Clark et al., 1999), topiramate (Aalbersberg and Mulder, 2006; Berlant, 2004), pregabalin, gabapentin, vigabatrin and tiagabine (Berigan and Arizona, 2002; Brannon et al., 2000; Connor et al., 2006; Macleod, 1996; Malek-Ahmadi, 2003) in reducing PTSD symptoms like nightmares, flashbacks, insomnia, irritability, impulsivity and violent behaviour. A double-blind, placebo-controlled study also showed that lamotrigine resulted in the improvement of re-experiencing and avoidance/numbing PTSD symptoms (Hertzberg et al., 1999).

The mood stabiliser lithium is the standard prophylactic drug for bipolar disorder, but is also effective against unipolar depression in conjunction with antidepressants (Bauer et al., 2000). Despite intensive research, the mechanism of action of lithium is poorly understood. Some of the proposed therapeutic actions of lithium include inhibition of glycogen synthase kinase-3β (GSK-3β) (Jope, 2004), reduction of inositol (Berridge et al., 1989), increasing the levels BCL-2 (Chen et al., 1999), decreasing levels of p53 and BAX (Chen and Chuang, 1999), and increasing activation of extracellular regulated kinase (ERK) and phosphoinositide-3 kinase (PI3K)-Akt (Chalecka-Franaszek and Chuang, 1999; Einat et al., 2003). Despite the proven efficacy of lithium in the treatment of mood disorders, literature on the efficacy of lithium in PTSD is limited. A case report (Forster et al., 1995) and open-label study (Kitchner and Greenstein, 1985) suggest that lithium may be helpful for improving anger, irritability and insomnia in patients with treatment-unresponsive PTSD.

In summary, anticonvulsants and mood stabilisers as pharmacotherapeutic treatment of PTSD is a promising area of research, but remains to be fully investigated.

2.4.1.8 Antipsychotics

Typical antipsychotics, such as haloperidol, clorpromazine, fluphenazine and thioridazine are believed to improve psychotic symptoms by blocking dopamine 2 (D₂) receptors. The second-generation, atypical antipsychotics including clozapine, olanzapine, quetiapine, risperidone and ziprasidone have diverse pharmacological actions, but with a common
antagonism of both D₂- and 5-HT₂A receptors (Scolnick, 2006). The finding that up to 40% of patients suffering from PTSD also display psychotic symptoms, indicates that antipsychotics may be effective in the treatment of PTSD and these drugs are indeed being used successfully (Ahearn et al., 2003).

Regarding the efficacy of typical antipsychotics in PTSD, a few case reports and an open-label trial suggest some benefit of thioridazine and fluphenazine (for example Dillard et al., 1993; Pivac and Kozarić-Kovačić, 2006). Due to their potentially serious side effects however, typical antipsychotics cannot be considered as a first-line treatment, but may be useful in a particular patient subtype with psychotic symptoms (Albucher and Liberonz, 2002).

The improved efficacy and side-effect profile of the second-generation or atypical antipsychotics over that of the typical antipsychotics have led to a renewed interest in the role of this class of medication in PTSD treatment. Case reports (for example Eidelman et al., 2000; Hamner, 1996; Sattar et al., 2002; Siddiqui et al., 2005), open-label studies (Hamner et al., 2003a; Monnelly et al., 2003; Petty et al., 2001) and double-blind placebo-controlled studies (Hamner et al., 2003b; Stein et al., 2002) suggest that clozapine, olanzapine, quetiapine, risperidone and ziprasidone may be effective as monotherapy or adjunct therapy for PTSD. In contrast, one small, double-blind study found no difference between olanzapine and placebo in the treatment of PTSD (Butterfield et al., 2001).

In summary, atypical antipsychotics may represent a valuable alternative pharmacotherapeutic choice in PTSD, although additional research on the subject is warranted.

2.4.2 Theory-Based Potential Treatments

The pharmacotherapy of PTSD discussed so far has been empirically based, with the pharmacological research consisting mostly of clinical trials with drugs initially developed for other disorders. As mentioned earlier, an alternative theoretical approach to pharmacotherapy of PTSD has also been suggested, involving the prediction of types of
pharmacological agents that might prove effective based upon the known, unique pathophysiology of PTSD (Friedman, 2000). Drugs identified on this theoretical basis include anti-adrenergic agents, narcotic agents, corticotrophin releasing hormone (CRH) antagonists, neuropeptide Y (NPY) enhancers, cholecystokinin (CCK) antagonists, substance P antagonists and glutamatergic drugs.

2.4.2.1 Anti-Adrenergic Agents

Stress activates the locus coeruleus (LC), which results in increased NE release in its projection sites, including the amygdala, hippocampus and prefrontal cortex (Bremner et al., 1996a). Furthermore, activation of the locus coeruleus also contributes to the sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis stimulation (Charney, 2004). Given the prominent role of the locus coeruleus-NE system in the stress response, it is not surprising that PTSD is associated with adrenergic dysregulation (Bremner et al., 1996b; Southwick et al., 1999a). Consequently, β-adrenergic blockers may reduce PTSD symptoms via dampening of peripheral NE drive. Indeed some (Famularo et al., 1998; Reist et al., 2001; Van der Kolk, 1983) but not all (Friedman and Southwick, 1995) case reports and pilot studies, propranolol effectively reduced re-experiencing and arousal symptoms, or re-emergent PTSD symptoms (Taylor and Cahill, 2002). Results from one non-randomised study and one double-blind, placebo-controlled pilot study, however, provides support for propranolol in suppressing PTSD symptoms and preventing the development of PTSD (Pitman et al., 2002; Vaiva et al., 2003).

Other adrenergic drugs tested in PTSD that attenuate NE activity include the α₂-adrenergic agonists, clonidine and guanfacine and the centrally active α₁-adrenergic antagonist, prazosin. Clonidine and guanfacine effectively reduced PTSD symptoms such as nightmares, insomnia, intrusive recollections, startle reactions and hypervigilance (for example Harmon and Riggs, 1996; Horrigan, 1996; Kinzie and Leung, 1989). Prazosin also reduced PTSD related nightmares (for example Peskind et al., 2003; Raskind et al., 2003; Taylor and Raskind, 2002) and psychological distress evoked by cues related to the trauma (Taylor et al., 2006).
In summary, several reports suggest efficacy for anti-adrenergic drugs in treating some PTSD symptoms, particularly nightmares and hyperarousal. In addition, preliminary results suggest efficacy for propranolol in the prevention of PTSD in the early aftermath of trauma. Since the prevention of PTSD is an important therapeutic goal, more studies on the exact role of propranolol in PTSD prevention are warranted.

2.4.2.2 Glutamatergic Drugs

Glutamate is the primary excitatory amino-acid (EAA) in the mammalian brain with approximately 60% of all neurones utilizing glutamate as their primary neurotransmitter (Javitt, 2004). Receptors for EAAs are divided into two main types, namely ionotropic and metabotropic. Ionotropic receptors include N-methyl-D-aspartate (NMDA)-, kainate- and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors which all control ion channels. Metabotropic receptors are coupled to G proteins and are divided into groups I, II and III according to second messenger coupling and ligand sensitivity (Ozawa et al., 1998). Glutamate, via its ionotropic and metabotropic receptors, plays a vital role in the regulation of several important central nervous system (CNS) processes including learning, memory, neuronal plasticity and cellular resilience (Mathew et al., 2005).

A growing body of evidence suggest involvement of the glutamatergic system in the pathophysiology and treatment of stress-related disorders (Zarate et al., 2003). For example, several mood and anxiety disorders, including depression and PTSD, are characterised by cognitive dysfunction and hippocampal shrinkage (Karl et al., 2006; Praag, 2004; Videbech and Ravnkilde, 2004). Together, the prominent role of glutamate in memory and learning and cognitive dysfunction in PTSD has led to the suggestion that brain glutamatergic systems may represent a crucial component of the disorder’s pathophysiology (Bonne et al., 2004). Indeed, fear conditioning and extinction, processes suggested to be involved in the neuropathology of PTSD, are both dependent upon proper functioning of NMDA receptors in the amygdala (Walker and Davis, 2002a). These findings have stimulated an interest for NMDA receptor modulating drugs in PTSD. However, NMDA receptors are involved in the acquisition, consolidation and extinction of memories (Abel and Lattal, 2001), making the choice of NMDA receptor agent...
difficult. To date, only one clinical pilot study on the use of NMDA receptor active drugs in PTSD has been performed. Heresco-Levy and colleagues (2002) reported that treatment of PTSD patients with the partial NMDA receptor agonist D-cycloserine resulted in significant improvements in numbing, avoidance, and anxiety symptoms. In summary, these data suggest a possible beneficial role for NMDA receptor regulating drugs in PTSD treatment. However, the nature and extent of NMDA receptor activation or inhibition is critical for the efficacy, side effects and toxicity.

Like NMDA receptors, AMPA receptors also participate in memory and learning and mediate the fast component of excitatory neurotransmission (Mathew et al., 2005). Clinical and experimental data suggest that positive modulation of AMPA receptors may be therapeutically relevant in mood disorders. This claim is supported by studies that show AMPA receptors to be responsive to chronic antidepressant treatment (Martinez-Turrillas et al., 2002; Svenningsson et al., 2002; Tan et al., 2006), as well as the antidepressant efficacy of positive allosteric modulators of AMPA receptors in preclinical studies (Knapp et al., 2002; Li et al., 2001). No clinical studies with AMPA modulators have, however, been performed in PTSD.

2.4.2.3 Other Theoretical Treatments

Several neuropeptides have been suggested to be involved in the stress response, including opioids, CRH, NPY, CCK and substance P (reviewed in Vermetten and Bremner, 2002a). Changes in these systems have also been detected in PTSD and may contribute to its pathophysiology (reviewed in Bremner et al., 1999; Vermetten and Bremner, 2002b). As a consequence, there has been interest in drugs that modulate some of these neuropeptides in the treatment of PTSD.

In this regard, preliminary data from pre-clinical and some clinical studies suggest that opioid agonists and antagonists (Bills and Kreisler, 1993; Glover, 1993; Lubin et al., 2002; Saxe et al., 2001), CRH antagonists (Habib et al., 2000), CCK-2 antagonists (Adamec et al., 1997; Cohen et al., 1999) and substance P antagonists (Kramer et al., 1998; Van der Hart et al., 2005) may be beneficial in the treatment of depression and PTSD.
2.5 Neurobiology

The mammalian brain filters and processes information from the internal and external environment, thereby allowing for the distinction between what is stressful and what is not, and for initiation of appropriate behaviour. In PTSD, however, the brain has a problem carrying out precisely these functions as a result of exposure to a traumatic stress (Van der Kolk, 1997).

2.5.1 Stress and the Fear Response

The term stress was originally used to describe a physical or emotional stimulus that disrupts the balance of the internal environment, and that may cause pathology when exceeding a critical level (Cannon, 1929). It has been recognised, however, that whether a potential stressor elicits a stress response depends not only on its level, but also on properties of the individual, such as assessment, coping style and social environment (Mason, 1975). Subsequently, stress has been redefined as a condition caused by a lack of information (uncertainty or unpredictability) about the outcome of a situation or stimulus (Levine and Ursin, 1991).

When exposed to a potential threat, the fear response is activated involving a series of coordinated responses in behaviour, autonomic- and endocrine function. The critical brain region involved in the stress- or fear response is the amygdala (Hamm and Weihe, 2005). On exposure to a threat or perceived threat, sensory stimuli are routed to the thalamus and conveyed directly and indirectly to the basolateral amygdala (Fendt and Fanselow, 1999). The short, direct route involves no cognition and results in the immediate activation of the fear response. Information travelling via the indirect, long route is processed by the cortex and hippocampus to determine the validity of the threat and subsequently, whether the fear response should be maintained, changed or discontinued (Bonne et al., 2004). Intra-amygdaloid circuitry connects the basolateral nucleus with the central nucleus, which sends projections to various centres involved in mediating fear associated automatic-, endocrine- and behavioural responses (Charney et al., 1995). Thus, the amygdala plays a critical role in processing fear-inducing stimuli and organising pro-adaptive responses (Charney et al., 1995), whereas the cortex and hippocampus process and integrate the
threatening stimulus into a logical, in-context representation and modulate the fear response accordingly (Vermetten and Bremner, 2002b).

The purpose of the fear response described above is to eliminate the source of the stress and reinstate homeostasis so that adaptation takes place and survival is promoted (Carrasco and Van de Kar, 2003). For example, the stress response prepares for fight or flight, and enhances the memory of the potentially life-threatening event, thereby optimising the response to similar events in the future (McEwen, 2000). Normally, reinstatement of the homeostatic balance follows successful adaptation. However, failure to cope with traumatic stress results in a new equilibrium, with significant neurobiological consequences. Chronic ongoing stress, as seen in PTSD, causes adaptive plasticity in the brain, in which neurotransmitters, neuropeptides and hormones interact to produce structural and functional changes (McEwen, 2000).

2.5.2 Neuroanatomy

The hippocampus, amygdala and prefrontal cortex have consistently been suggested to be involved in the pathophysiology of PTSD and although other brain regions may also be involved, the following discussion will focus only on these three structures.

2.5.2.1 Hippocampus

With regard to the hippocampus, many (Bremner et al., 2003a; Lindauer et al., 2004b; Shin et al., 2004b) but not all (Freeman et al., 2006; Golier et al., 2005; Jatzko et al., 2006) studies have found a smaller hippocampal volume in PTSD patients compared to controls. In an attempt to clarify these discrepant findings, several meta-analyses of these and other studies have been performed and all report a significant decrease in left and right hippocampal volume in adult PTSD (Karl et al., 2006; Kitayama et al., 2005; Smith, 2005). Mixed findings have also been reported with regard to decreased hippocampal neuronal integrity (Chen et al., 2006; Freeman et al., 2006; Li et al., 2006a; Yang et al., 2006).
Decreased hippocampal volume and neuronal integrity in PTSD is consistent with the view that exposure to stressful experiences and subsequent PTSD development leads to hippocampal atrophy, which can be reversed with successful SSRI treatment (Bremner and Vennetten, 2004; Vermetten et al., 2003). It should be mentioned however, that several studies have brought into question the exact relationship between hippocampal volume and PTSD. Studies in the acute aftermath of trauma (Karl et al., 2006; Winter and Irle, 2004) or in monozygotic twins (Gilbertson et al., 2002; Pitman et al., 2006) suggest that decreased hippocampal volume may be associated with trauma exposure or a pre-trauma risk factor, respectively. Thus, changes in hippocampal structure may not necessarily be an expression of PTSD pathophysiology, a hypothesis that is supported by the lack of change in hippocampal volume after successful psychotherapy (Lindauer et al., 2005).

Finally, functional imaging studies consistently demonstrate impaired hippocampal activation during a declarative memory task and significant decreased hippocampal blood flow during retrieval of memories of abuse (Bremner et al., 1999a; Bremner et al., 2003a; Bremner et al., 2003b).

### 2.5.2.2 Amygdala

Similar to the hippocampus, meta-analysis of imaging studies also indicate a decrease in amygdala volume bilaterally (Karl et al., 2006). It should be mentioned, however, that although these effects were significant, they were relatively small, especially compared to those seen for the hippocampus (Karl et al., 2006). In contrast, functional imaging studies suggest a robust increase in amygdala response in PTSD patients compared to controls after exposure to reminders of traumatic events or fearful stimuli (Hendler et al., 2003; Pissiota et al., 2002; Protopopescu et al., 2005; Shin et al., 1997; Shin et al., 2005).

### 2.5.2.3 Prefrontal Cortex

Although adult PTSD does not appear to be associated with structural changes in the prefrontal cortex (Karl et al., 2006), some changes in the volume of specific subregions of the prefrontal cortex subregions have been found in paediatric PTSD (Richert et al.,
2006). Adult PTSD is, however, associated with significantly attenuated responses within the medial prefrontal cortex in response to reminders of traumatic events or fearful stimuli (Bremner et al., 1999c; Bremner et al., 1999a; Lanius et al., 2001; Lindauer et al., 2004a; Shin et al., 1999; Shin et al., 2004a; Shin et al., 2005).

2.5.3 Neurocircuitry

As discussed above, various structural and functional changes in the hippocampus, amygdala and prefrontal cortex have been found in PTSD. Given the important role of these brain regions in the fear/stress response, emotion and cognition, it is not surprising that alterations in their structure and/or responsivity in PTSD have been suggested to have clinical consequences.

A recently proposed neurocircuitry model of PTSD suggests that hyperresponsivity of the amygdala may underlie exaggerated fear conditioning (section 2.5.4.1) and explain symptoms of hyperarousal and the permanent quality of emotional memory of the trauma. Moreover, the model postulates that inadequate inhibition of the amygdala by the prefrontal cortex may explain deficits in extinction and attention, and mediate persistent recall of traumatic memories and deficits in working memory. Finally, the model suggests that decreased hippocampal function may underlie deficits in identifying safe stimuli and mediate generalisation and cross-sensitisation (section 2.5.4.3) of environmental stimuli, as well as dysfunction of explicit memory (Bonne et al., 2004; Liberzon and Martis, 2006; Rauch et al., 2006).

2.5.4 Psychobiological Models

The primary symptoms of PTSD - re-experiencing, avoidance/numbing and hyperarousal - are related to neuronal mechanisms involved in fear and memory (Charney, 2004).

Memory is typically divided into declarative and non-declarative memory. Declarative memory includes the recall of factual knowledge and is mediated by the hippocampus and prefrontal cortex. Non-declarative memory includes skills learning and emotional learning and is mediated mainly by the amygdala (Elzinga and Bremner, 2002). PTSD is
characterised by a cognitive paradox, with disturbances in both declarative and non-declarative memories. On the one hand, there is an extraordinarily strong memory of the traumatic event and on the other, the patient experiences amnesia for certain aspects of the encounter. Furthermore, patients suffering from PTSD also display impairment in the ability to store and retrieve new information (Van Praag, 2004).

In addition to a memory disorder, PTSD is also considered to be a fear disorder. Trauma not only leads to the formation of memories of the event, but also sensitises the individual, thereby increasing the general responsiveness to potentially harmful stimuli (Siegmund and Wotjak, 2006).

Fear conditioning, failure of extinction, increased reconsolidation, sensitisation, cross-sensitisation, kindling, learned helplessness, and cognitive processing deficits have all been proposed to contribute to the three core symptoms of PTSD (Bonne et al., 2004; Charney, 2004).

### 2.5.4.1 Fear Conditioning

In PTSD, autonomic arousal, vivid memories and even flashback can be elicited by external or internal, often unremarkable stimuli (Bonne et al., 2004). The emotional response is highly stressful and as a result, numbing of general emotional responsiveness may occur as patients try to avoid these stimuli in everyday life (Charney et al., 1995). One psychobiological mechanism that may account for the association between traumatic re-experiencing symptoms and harmless stimuli is fear conditioning (Charney, 2004).

In an animal, when a non-threatening stimulus is presented together with an aversive stimulus, the animal rapidly exhibits a fear response to the presentation of the non-threatening stimulus alone (Bonne et al., 2004). Fear conditioning is a positive adaptive mechanism in life-threatening situations, optimising an animal’s response and attention to danger (Charney, 2004; Sanders et al., 2003). In contrast, fear conditioned responses in PTSD are maladaptive and there may be a loss of stimulus discrimination. Patients suffering from the disorder may therefore display re-experiencing and hyperarousal symptoms as a result of both threat- and non-threat related stimuli (Bonne et al., 2004).
One manifestation of loss of stimulus discrimination is an abnormal startle response, a sequence of muscular and autonomic responses elicited by sudden and intense stimuli (Van der Kolk, 2001). In human studies, startle is typically measured from eye-blink responses and some (Butler et al., 1990; Morgan et al., 1996; Orr et al., 1995; Shalev et al., 1997) but not all studies (Metzger et al., 1999; Orr et al., 1997; Shalev et al., 1992; Shalev et al., 2000) provide support for increased eye-blink startle responses in patients with PTSD. Failure of habituation and decreased pre-pulse inhibition in PTSD has also been reported (Grillon et al., 1996; Shalev et al., 2000).

The psychobiological model of fear conditioning in PTSD is supported by clinical studies that show increased skin conductance and heart rate responses to the conditioned stimulus to occur to a greater extent in PTSD patients compared to trauma-exposed controls (Orr et al., 2000). Therefore, therapies targeting fear conditioning may be clinically useful in the treatment of stress-induced disorders. In this regard, the NMDA receptor antagonist memantine may prevent PTSD (Charney, 2004). Another approach to prevent PTSD may be reducing the impact and intensity of recently acquired fear memory by using clinically available Ca\(^{2+}\) channel blockers such as verapamil and nimodipine (Charney, 2004). Other drugs that may possibly prevent PTSD in a similar way are opioid agonists (Saxe et al., 2001) and adrenergic receptor antagonists (Pitman et al., 2002; Vaiva et al., 2003).

### 2.4.3.2 Extinction and Reconsolidation

**Extinction**

Memories, including fear memories, are not only vulnerable after learning, but also after retrieval. Retrieval of a memory can lead to two opposing processes, one that weakens the old memory (extinction) and one that strengthens it (reconsolidation) (Eisenhardt and Menzel, 2007). Abnormalities in both extinction and reconsolidation have been proposed as psychobiological models in PTSD (Bonne et al., 2004).

The fear response system may be activated by previously harmful stimuli after they were paired with negative effects (Hamm and Weike, 2005), enabling the anticipation and efficient response to environmental dangers. However, when signals for aversive events
no longer predict those events, the previously learned conditioned emotional fear
response to those signals is reduced and eventually disappears (Maren and Quirk, 2004).
This process is known as extinction, which may involve the learning of new associations
that compete with or override the original, fear response producing associations
(Anderson and Insel, 2006; Charney et al., 1995). Thus, extinction of fear is considered
inhibitory learning, which prevents the expression of the fear response to old associations,
rather than erasing those associations. Because the original associations remain intact,
fear conditioning can be reinstated to pre-extinction level by re-representation of the
unconditioned stimulus (Barad et al., 2006).

Many PTSD patients describe persistent recall of traumatic memories with accompanying
fear responses, and reduced extinction of conditioned responses has been suggested to
underlie this phenomenon (Bonne et al., 2004; Peri et al., 1999). In addition, the ability of
conditioned fear to be reinstated may explain the clinical observation that dormant
traumatic memories may be brought out by a subsequent stressor or by a stimulus
associated with the original trauma (Charney et al., 1995). Moreover, the leading
vulnerability factor for PTSD is past history of trauma and childhood abuse, suggesting
that re-exposure to a traumatic event even after extinction of the original fear response,
could trigger PTSD (Bonne et al., 2004).

Clinical studies show PTSD patients to have reduced extinction of fear conditioned
responses (Peri et al., 1999) and extinction learning forms the basis of exposure
psychotherapies for clinical conditions associated with exaggerated fear responses such as
PTSD (Foa, 2000). Therefore, methods of facilitating extinction may improve current
therapeutic interventions. In this regard, the partial NMDA receptor agonist D-cycloserine
has been proposed as additional pharmacological treatment in PTSD (Davis, 2002;
Heresco-Levy et al., 2002; Lee et al., 2006c). In contrast, drugs that attenuate extinction
may be counterproductive during exposure therapy. In this regard, benzodiazepines often
prescribed for their utility in the treatment of bouts of extreme anxiety, may hinder
exposure therapy (Davis and Myers, 2002).
Reconsolidation

As mentioned earlier, when an old memory is recalled, it can either be weakened by extinction learning, or strengthened by reconsolidation. Repeated activation of traumatic memories may serve to strengthen them and each time a traumatic memory is retrieved, it is integrated into an ongoing emotional experience and becomes part of a new memory. Therefore, increased reconsolidation is extremely relevant to PTSD and has been suggested to contribute to persistent trauma-related symptoms (Bonne et al., 2004; Charney, 2004).

The reconsolidation process can be disrupted and this is clinically relevant in memory disorders (Lee et al., 2006). In PTSD for example, noradrenergic blockade may be useful in attenuating traumatic memories, even well-consolidated old memories (Debiec and Le Doux, 2006).

Despite the promising clinical benefits of drugs that affect extinction or reconsolidation, it should be kept in mind that because the two processes use the same neurotransmitter systems, a drug that disrupts reconsolidation may also impair extinction and a drug that enhances reconsolidation may also impair extinction. For example, both processes make use of NMDA receptors and it has been shown that while MK-801 disrupts reconsolidation, it also impairs extinction. Similarly, while D-cycloserine may enhance extinction, it also enhances reconsolidation (Lee et al., 2006). Caution should therefore be exercised when choosing drugs to enhance exposure therapies in PTSD.

2.5.4.3 Other Models

Sensitisation, cross-sensitisation, kindling, learned helplessness and deficits in cognitive processing have also been proposed as psychobiological models of PTSD (Bonne et al., 2004; Charney et al., 1993; Charney et al., 1995; Hageman et al., 2001).

Sensitisation generally refers to the increase in the magnitude of behavioural, neurophysiological or pharmacological responses, following exposure to an environmental or pharmacological stimulus (Charney et al., 1993). Sensitisation may be
adaptive, providing the capability to respond more readily or effectively to subsequent stimuli and thereby increasing the probability of survival (Stam et al., 2000). However, sensitisation may also be maladaptive, leaving the organism hypervigilant, overreacting to minor stimuli and continuing to act as if danger exists even when no danger is present (Southwick and Friedman, 2001). In addition, cross-sensitisation may occur, involving an augmented response to a different stimulus than the original response inducing stimulus (Charney et al., 1993).

Kindling refers to the phenomenon of an increase in convulsive response as a result of repeated, intermittent stimulation of the brain (Goddard et al., 1969). The kindling hypothesis might also have relevance to mood and anxiety disorders, where certain life events after one or more episodes of illness would kindle limbic nuclei and make way for new episodes (Hageman et al., 2001; Post and Weiss, 1998).

Learned helplessness is a behavioural response to inescapable, uncontrollable, continuous stress. This response is adaptive for animals in situations where fight and flight reactions are unsuccessful. For example, when confronted by an inescapable predator, complete inactivity may cause the predator to lose interest (Bonne et al., 2004). Learned helplessness is valid as a model of PTSD, since a person’s response to a traumatic event should include “helplessness, fear or horror” for a diagnosis to be made (APA: DSM-IV-TR, 2002).

Evidence from event-related brain potential studies suggest cognitive processing abnormalities in PTSD, with disturbances in the ability to filter out irrelevant information, preconscious sensitivity to stimulus change, cortical responsiveness to increasing stimulation, orientating processes and attentional processes involved in the evaluation of neutral and trauma-relevant information (Orr et al., 2002).

Sensitisation and kindling may explain delayed onset PTSD, persistence of PTSD symptoms long after the initial trauma, the worsening of the disorder over time and the negative effect of early life adversity on subsequent responses to stressful life events (Charney et al., 1995; Charney et al., 1993; Hageman et al., 2001; Smid et al., 2003). Cross-sensitisation may explain the loss of stimulus discrimination sometimes seen in PTSD, where patients increasingly react to both threat-related and non-related stimuli,
resulting in constant anxiety (Bonne et al., 2004). Learned helplessness may underlie avoidance symptoms of PTSD, where intrusive recollections of the trauma become an inescapable stressor, leading to learned helplessness-like behaviour such as withdrawal, diminished interest and a sense of a limited future (Bonne et al., 2004). Finally, clinical manifestations of cognitive processing abnormalities may include hypervigilance and attentional/concentration difficulties (Orr et al., 2002).

2.5.5 Neurochemistry

The central components of the stress system are the CRH neurones and LC/noradrenergic neurones of the hypothalamus and brain stem. CRH and LC/noradrenergic neurones regulate the peripheral activities of the HPA axis and the sympathetic nervous systems, respectively (Elenkov and Chrousos, 1999). In addition to CRH and NE, a number of other neurotransmitters, neuropeptides and hormones are released upon exposure to a traumatic stressor. Together, these stress mediators are adaptive, functioning to help an individual deal with stress.

Normally, stress produces rapid responses in hormonal levels, which return to baseline levels after the threat has passed. However, failure to cope with traumatic stress causes changes in local neurotransmitters and systemic hormones in an attempt to reach a new equilibrium. Over time, the failed shut-off of stress mediators has a negative effect on the body, called "allostatic load" (McEwen, 2003). In PTSD, allostatic load can take the form of neurochemical imbalance. In this regard, changes in the CRH/HPA axis and LC/noradrenergic system, as well as in other neurotransmitters, neuropeptides, hormones, and immune system factors associated with PTSD will be discussed in the following section.

2.5.5.1 CRH/HPA axis

The HPA axis plays an important role in the stress response. In response to certain internal and external (stress) signals, neurones in the hypothalamic paraventricular nucleus synthesise CRH, as well as other factors such as arginine vasopressin (AVP). These hormones are secreted into the hypophysial portal circulation and transported to the
pituitary, where they act on specific receptors to trigger the release of adrenocorticotripin (ACTH) into the systemic circulation. ACTH then stimulates type 2 melanocortin receptors in the adrenal cortex to initiate the synthesis and systemic release of glucocorticoids such as cortisol and corticosterone. Cortisol is the main glucocorticoid in man, whereas rodents produce more corticosterone (Buckingham, 2006; Herman et al., 2005).

**Glucocorticoids**

Glucocorticoids mediate their effects through two distinct intracellular receptors, namely type I and type II receptors. The type II receptor, also known as the glucocorticoid receptor (GR), is highly expressed throughout the body, including the brain. Glucocorticoids have a relatively low affinity for GRs and as a result, these receptors are extensively bound only during periods of intermediate to high glucocorticoid secretion (Buckingham, 2006; Herman et al., 2005). The type I receptor, also known as the mineralocorticoid receptor (MR), has a more limited distribution than GRs, and is expressed in some areas of the brain, most notably the limbic system, entorhinal cortex and, to a lesser extent, the hypothalamus. Due to its high affinity for glucocorticoids, the MR is extensively bound during periods of basal secretion (Buckingham, 2006; Herman et al., 2005). Both type I and II receptors are believed to mediate their effects by slow, genomic mechanisms, as well as fast, non-genomic mechanisms (Makara and Haller, 2001).

Glucocorticoids have widespread actions throughout the body and help prepare for and respond to stress and importantly, to hold back the stress-activated defence reactions and prevent their overactivation (Sapolsky et al., 2000). In this regard, glucocorticoids have been shown to affect the cardiovascular-, immune-, reproductive- and metabolic systems, as well as the central nervous system. Neurobiological effects of glucocorticoids include the complex modulation of memory, centred in the hippocampus (Sapolsky et al., 2000). Basal levels enhance learning via activation of MRs, whereas stress levels of glucocorticoids acting via GRs disrupt learning (Sapolsky, 2003; Wolf, 2003). Prolonged exposure to stress levels of glucocorticoids may also lead to inhibition of dentate gyrus neurogenesis, dendritic atrophy in the hippocampal CA3 region, endangerment of hippocampal neurones and hippocampal neuron toxicity (Radley and Morrison, 2005;
Sapolsky, 1999). Although the exact mechanisms by which glucocorticoids exert these deleterious effects on the brain are unknown, disruption on glucose uptake and utilisation, exacerbation of glutamate and free radical toxicity, and disruption of cellular defences have all been implicated (De Kloet et al., 1998; Reagan and McEwen, 1997; Sapolsky, 1999).

**CRH**

In addition to its key role in the activation of the HPA axis, CRH also modulates neuronal activity, acting as a neurotransmitter and neuromodulator to mediate the behavioural response to stress (Deussing and Wurst, 2005). In this regard, CRH-containing neurones have been found throughout the brain, including the amygdala, hippocampus and prefrontal cortex (Arzt and Holsboer, 2006; Smagin et al., 2001).

The biological activity of CRH and CRH-related peptides (urocortin 1, urocortin 2, urocortin 3) is mediated by two receptors, CRH₁ and CRH₂. CRH is relatively selective for CRH₁ receptors compared to CRH₂ receptors, urocortin 1 binds both receptors with high affinity, and urocortin 2 and -3 bind selectively and with high affinity to CRH₂ receptors (Lowry and Moore, 2006). In rodents, CRH₁ receptors are expressed in the pituitary and throughout the brain, whereas CRH₂ receptor expression is limited to particular brain regions and some peripheral organs (Deussing and Wurst, 2005). A more widespread CRH₂ receptor expression has however, been described in monkeys (Arzt and Holsboer, 2006). CRH receptors are G-protein coupled receptors (GPCRs) linked to several intracellular signalling pathways (Dautzenberg and Hauger, 2002).

Preclinical studies indicate a role for CRH in a host of behavioural processes including learning and memory, arousal, startle, fear, anxiety and general motor activity (Radulovic et al., 1999; Risbrough, et al., 2004; Takahashi, 2001; Spina et al., 2000; Van Gaalen et al., 2002). CRH may mediate these effects by interacting with monoaminergic systems, as amygdala CRF terminals have been shown on CRH₁ expressing cells in the NE-producing LC, the 5-HT producing dorsal raphae and the dopamine (DA) producing ventral tegmental area (Bale, 2005).
Regulation of the HPA axis

The sensitivity of the HPA axis to incoming stimuli is regulated by a negative feedback system. Endogenous cortisol binds to corticoid receptors in components of the HPA axis and acts as a potent negative regulator of HPA activity by inhibiting the release of CRH/AVP and ACTH (Buckingham, 2006). There is also evidence that cortisol indirectly inhibits the hypothalamus, via other brain regions such as the hippocampus, prefrontal cortex and medial preoptic area (Ziegler and Herman, 2002). For example, cortisol stimulation of MRs in the hippocampus may inhibit hypothalamic CRH release and maintain the tone of basal HPA axis activity (De Kloet et al., 1998; Michelson et al., 1995). The magnitude of the HPA response to stress thus depends upon the pre-existing glucocorticoid tone, and a deficiency in MR activation may allow more readily a corticosterone response and more pronounced GR-mediated effects (Buckingham, 2006).

The sensitivity of the HPA axis to glucocorticoid feedback is not constant and when exposed to chronic stress, can show both response 'habituation' and 'facilitation'. The mechanisms underlying this adjustment of feedback sensitivity may involve changes in GR and MR density or function (Herman et al., 2005). In this regard, it has been suggested that MRs maintain neuronal homeostasis and limit the disturbance by stress, whereas GRs help the recovery after stress and stores the experience for responses to future encounters. An imbalance in MR/GR-mediated actions may therefore result in HPA axis dysregulation and maladaptive behaviour (De Kloet and Derijk, 2004).

It should be kept in mind that in addition to glucocorticoid feedback regulation, the HPA axis is also susceptible to glucocorticoid-independent inhibition from neuronal sources (Herman et al., 2005; Ziegler and Herman, 2002). In this regard, secretory cells within the paraventricular nucleus receive neuronal inputs from a number of brain regions including the amygdala, hippocampus, and midbrain nuclei (Young, 2004). The innervation of the paraventricular nucleus is mainly GABA-ergic and together with the expression of GABA_A receptor subunits in this region, suggests an inhibitory role for GABA in HPA axis regulation (Ziegler and Herman, 2002).

In summary, glucocorticoid-dependent and -independent regulatory mechanisms are important in determining basal levels and circadian changes in cortisol levels. Together,
these mechanisms ensure that the extent and duration of the glucocorticoid response to stress is limited and proportionate to the stressful challenge (Ziegler and Herman, 2002).

**CRH/HPA axis Alterations in PTSD**

As expected in a stress-related disorder, most studies in PTSD patients have found significant CRH/HPA axis alterations. In addition, some of these neuroendocrinological findings seem to be unique to PTSD.

Although many studies show decreased urinary basal cortisol levels in PTSD (Glover and Poland, 2002; Mason et al., 1986; Thaller et al., 1999; Yehuda et al., 2000), there have been reports of no changes (Baker et al., 1999; Rasmussen et al., 2001) or even increased basal levels (De Bellis et al., 1994; Maes et al., 1998). Similar mixed results have been obtained for basal salivary cortisol levels (Boscarino, 1996; Inslicht et al., 2006; Kellner et al., 1997; Young and Breslau, 2004). These discrepancies may be due to methodological variations between studies, or alternatively, may indicate that low cortisol levels are particular to trauma victims with early life stress, specific features of PTSD, or specific subtypes of PTSD (Yehuda, 2003; Yehuda, 2006).

Studies of circadian parameters of cortisol in PTSD have revealed reduced cortisol levels at several time points (Yehuda et al., 1996) and an increased range of cortisol levels over time (Thaller et al., 1999). These data imply that in PTSD, the HPA axis may have a low background activity and therefore, the ability to respond maximally to environmental stimuli (Yehuda, 2003).

In an attempt to clarify the role of the HPA axis in PTSD, prospective studies have investigated the relationship between cortisol and the disorder. Some of these studies suggest that the acute cortisol response to a trauma may be blunted in persons who eventually develop PTSD (Delahanty et al., 2000; McFarlane et al., 1997). Results from another prospective study, however, point to the possibility that low a cortisol level is a risk factor for developing PTSD, rather than a consequence of trauma or the pathophysiology of the disorder (Yehuda et al., 2000; Yehuda, 2003).
With regard to other mediators of the HPA axis, studies have demonstrated elevated cerebrospinal CRH levels in PTSD (Baker et al., 1999; Bremner et al., 1997), but no alterations in plasma ACTH levels, even in when cortisol levels were reduced (Kanter et al., 2001; Kellner et al., 1997).

The combination of high baseline CRH levels and low baseline plasma cortisol levels in patients with PTSD are in contrast with results seen in major depressive disorder, which generally presents with elevated baseline CRH and cortisol ( Claes et al., 2004; Gillespie et al., 2005). In an attempt to explain this unique pattern of HPA axis alterations in PTSD, numerous studies have used pharmacological and non-pharmacological challenge paradigms to test several hypotheses, including enhanced feedback sensitivity to cortisol, pituitary insufficiency or reduced sensitivity, and adrenal insufficiency or reduced sensitivity (De Kloet et al., 2006).

The majority of non-pharmacological stress challenge tests have found increased cortisol levels in the anticipation phase and during the challenge (Bremner et al., 2003c; Elzinga et al., 2003). These results provide support for the hypothesis that the HPA axis is maximally responsive to stress-related stimuli in PTSD (Yehuda, 2003), but is in contrast with the hypotheses of pituitary and adrenal insufficiency as the main cause for low cortisol baseline levels in PTSD (De Kloet et al., 2006).

CRH challenge studies designed to test pituitary responsivity in PTSD have been inconclusive (Kellner et al., 2003; Rasmusson et al., 2001; Smith et al., 1989). Results from studies employing the low dose dexamethasone challenge test (Lindley et al., 2004; Newport et al., 2004; Yehuda et al., 1995; Yehuda et al., 2002), however, support the hypothesis of pituitary insufficiency or reduced sensitivity in PTSD (De Kloet et al., 2006). Results from studies using the fast ACTH challenge or the metyrapone test generally fail to support the hypotheses of adrenal insufficiency or reduced sensitivity in PTSD (De Kloet et al., 2006; Rasmusson et al., 2001). Finally, pharmacological challenge studies have also investigated GR and MR function in PTSD. Enhanced sensitivity of GRs to dexamethasone in cells of the immune system has been observed (Yehuda et al., 2004), providing support for the hypothesis of enhanced negative feedback in PTSD (De Kloet et al., 2006). No differences in MR function between PTSD patients and healthy controls have been found (Kellner et al., 2002; Otte et al., 2006).
Clinical Relevance

PTSD presents with unique neuroendocrine alterations, notably low baseline cortisol, high baseline CRH, and exaggerated cortisol response to stress challenges. It should be mentioned, however, that with very few exceptions, the cortisol levels reported in the literature for patients with PTSD are within the normal range. Nevertheless, cortisol alterations within the normal range may still have clinical significance, although this has not yet been fully established (Yehuda, 2006).

It is also not yet clear whether low baseline cortisol represents an abnormal acute stress response at the time of the trauma, the pathophysiology of PTSD, or a pre-existing risk factor for the disorder. Nevertheless, given the important role of glucocorticoids and CRH in preparing for, mediating and terminating the stress response, dysregulation of the HPA axis (before, during or after the trauma), may have a severe negative impact on an individual's ability to respond to and cope with stress. In this regard, low levels of cortisol may disrupt the inhibition of the stress response by failing to inhibit pituitary activation and CRH secretion and prolonging NE availability to synapses. Ultimately, low cortisol may also result in an increase in the sensitivity of GRs, further strengthening negative feedback and lowering cortisol levels even more. Alternatively, enhanced negative feedback may already be present at the time of the trauma, thereby contributing to premature suppression of cortisol secretion (Yehuda, 2003).

The glucocorticoid cascade hypothesis suggests that the prolonged overproduction of glucocorticoids as a result of ongoing stress damages brain structures essential for HPA axis restraint, especially the hippocampus (Sapolsky et al., 1986). However, in addition to glucocorticoid excess, insufficient glucocorticoid signalling may also play a significant role in the development and expression of stress-related disorders. Adaptive changes such as increased GR sensitivity may compensate for the observed hypocortisolism in PTSD (Raison and Miller, 2003). Chronic low levels of cortisol may disrupt the MR/GR balance, compromising neuronal homoeostasis (De Kloet and Derijk, 2004). Given the importance of MR activation in memory function (Wolf, 2003), it has been suggested that reduced cortisol levels, especially during sleep, may play a role in both the overconsolidation of emotional memories and the impaired declarative memory seen in PTSD (Wagner et al., 2005). In addition, it has been argued that MR activation maintains
the balance between cell birth and cell death in the dentate gyrus and that chronic lack of cortisol may cause neurodegeneration and synaptic function loss of this region (De Kloet et al., 1998). In this regard, adult PTSD has been associated with significantly smaller hippocampal volume compared with control subjects (Kitayama et al., 2005; Smith, 2005). Finally, hypocortisolism may also lead to inadequate glucocorticoid-mediated feedback inhibition of immune responses, leading uncontrolled release of pro-inflammatory cytokines. In this regard, PTSD has been associated with increased secretion of interferon (IFN)-γ, interleukin (IL)-1β, and IL-6 (Maes et al., 1999a; Spivak et al., 1997; Tucker et al., 2004b; Woods et al., 2005). These pro-inflammatory cytokines may induce a syndrome of “sickness behaviour”, which share many symptoms with PTSD, including sleep alterations and cognitive dysfunctions (Raison and Miller, 2003).

In addition to low baseline levels, studies have shown exaggerated cortisol response in PTSD patients during stressful reminders of their trauma (Bremner et al., 2003c; Elzinga et al., 2003). Thus, PTSD patients may be intermittently exposed to high levels of cortisol over the course of their illness, possibly leading to impaired hippocampal neurogenesis, neuronal functioning and survival, and disrupted memory processes (Radley and Morrison, 2005; Sapolsky, 1999; Wolf, 2003). Thus, in PTSD, the initial release of cortisol may facilitate the formation of long-lasting traumatic memories, whereas the repeated exaggerated cortisol response to reminders of the trauma may cause hippocampal atrophy and disruption of memory process. In this regard, PTSD is associated with significantly smaller hippocampal volume (Kitayama et al., 2005; Smith, 2005), and patients often present with impaired ability to store and retrieve new information (Van Praag, 2004).

Finally, studies have demonstrated increased basal levels of cerebrospinal CRH levels. CRH plays an important role in the stress response by modulating behaviours such as learning and memory, arousal, startle, fear, anxiety and general motor activity (Radulovic et al., 1999; Risbrough, et al., 2004; Takahashi, 2001; Spina et al., 2000; Van Gaalen et al., 2002). Activation of CRH₁ receptors alters the neurotransmission of DA, 5-HT and NE in brain regions associated with emotions and mood, and it is therefore not surprising that CRH is believed to play a role in the development of anxiety and mood disorders (Bale, 2005). In PTSD, increased basal CRH levels may be responsible for sustained fear and anxiety, increased startle reactivity and disruption of pre-pulse inhibition (Risbrough
and Stein, 2006; Schulkin et al., 2005). These effects may be mediated by the interaction of CRH with monoamines, especially NE, which has been suggested to exist in a feed-forward system with CRH (Koob, 1999; Linthorst, 2005).

2.5.5.2 The Locus Coeruleus/Noradrenergic System

The LC/noradrenergic system plays an important role in the stress response. A variety of intrinsic and extrinsic stressors are able to activate the LC through projections from sensory relay areas, leading to NE release in projection sites including the amygdala, hippocampus, hypothalamus and prefrontal cortex (Charney, 2004). NE acts at multiple receptors in target tissues and the pharmacologic classification of noradrenergic receptors includes alpha (α1 and α2) and beta (β1, β2 and β3) adrenergic receptors (Carrasco and Van de Kar, 2003). The distribution and second messenger coupling of these receptor subtypes vary within and across brain regions (Berridge and Waterhouse, 2003).

The LC/noradrenergic system primarily influences arousal (Haden and Scarpa, 2007) and plays a critical role in the level of alertness, vigilance, orientation, selective attention, memory, and cardiovascular responses to life-threatening stimuli (Southwick et al., 1999a). Thus, it serves as a general warning system and assists the response to relevant stimuli and suppresses the response to irrelevant stimuli (Berridge and Waterhouse, 2003; Haden and Scarpa, 2007; Vermetten and Bremner 2002a). In this regard, dysregulation of the LC/noradrenergic system may be involved in PTSD (Singewald and Philippu, 1998).

Noradrenergic Alterations in PTSD

Most studies of baseline physiologic arousal have reported similar levels of heart rate, blood pressure, and skin conduction in patients with PTSD compared to control subjects (Casada et al., 1998), with some negative findings (Forneris et al., 2004; Southwick et al., 1999b). Similarly, exaggerated baseline startle reflexes in individuals suffering from PTSD have been reported in some (Butler et al., 1990; Morgan et al., 1996) but not all studies (Grillon et al., 1996). Similarly, most (De Bellis et al., 1997; Spivak et al., 1999; Yehuda et al., 1998) but not all (Mellman et al., 1995; Pitman and Orr, 1990) studies have found increased urinary excretion of epinephrine and NE in PTSD patients compared to
controls. Some studies also show increased basal plasma levels of epinephrine and NE in PTSD patients compared to controls (Spivak et al., 1999; Yehuda et al., 1998), while others do not (Geracioti et al., 2001; McFall et al., 1992). Finally, significantly higher baseline cerebrospinal fluid (CSF) NE levels have been reported in patients with PTSD (Geracioti et al., 2001).

In contrast to baseline studies, heightened sympathetic activity (Carson et al., 2000; Casada et al., 1998; McDonagh-Coyle et al., 2001; Orr et al., 2003; Pitman et al., 2001), exaggerated startle reflexes (Grillon et al., 1998) and increased noradrenergic responses (Blanchard et al., 1991; McFall et al., 1990) in response to trauma-related cues have been consistently reported in PTSD.

Finally, pharmacological challenges with yohimbine, an $\alpha_2$-adrenergic autoreceptor antagonist, results in panic attacks, flashbacks, increased plasma NE metabolite levels (MHPG), and increased startle responsivity in PTSD subjects compared to controls (Morgan et al., 1995; Southwick et al., 1993), suggesting an exaggerated response to adrenergic stimulation in the disorder.

**Clinical Relevance**

Enhanced noradrenergic functioning may be responsible for both hyperarousal and re-experiencing symptoms in PTSD. Increased noradrenergic activity in brain areas involved in memory formation, such as the amygdala and hippocampus, may result in increased learned fear responses and consolidation of fear memories. In addition, uninhibited noradrenergic stimulation may decrease the inhibitory influence of the prefrontal cortex on the amygdala, thereby contributing to the acquisition and maintenance of fear conditioning due to increased consolidation and decreased extinction (O’Donnell et al., 2004).

**2.5.5.3 The Serotonergic System**

The vast majority of central serotonergic neurones are located in the dorsal and median raphé nuclei within the brain stem. Together, these two nuclei innervate several brain
regions including the hippocampus, amygdala and several hypothalamic nuclei (Lechin et al., 2006). Serotonergic neurones possess diverse morphology and subpopulations have unique functional and behavioural roles (Lowry, 2002). In this regard, the serotonergic system has been shown to be involved in anxiety, arousal, vigilance, aggression, mood and impulsivity, as well as the regulation of sleep and food intake (Raymond et al., 2001; Vermetten and Bremner, 2002a). In addition, serotonergic neurones have a major influence on the regulation of neuroendocrine function and the stress-response. In this regard, serotonergic systems may contribute to facilitation or inhibition of basal and stress-induced glucocorticoid secretion, depending on the nature of the stressor (Lowry, 2002). Finally, 5-HT functions to dampen noradrenergic neuronal firing in the LC (Newport and Nemeroff, 2000; Singewald and Philippu, 1998).

5-HT exerts its effects via 7 subfamilies of receptors (5-HT1-7), each capable of mediating different and sometimes opposite effects (Raymond et al., 2001). Given its role in anxiety and the stress-response, it is not surprising that dysregulation of the serotonergic system has been suggested to play a role in mood disorders, as well as anxiety disorders such as PTSD (Carrasco and Van de Kar, 2003).

**Serotonergic Alterations in PTSD**

Platelet serotonin uptake as measured by paroxetine binding has been found to be significantly decreased (Arora et al., 1993; Maes et al., 1999b) or unchanged (Mellman and Kumar, 1994) in PTSD patients compared to healthy controls. The former finding suggests a defect of the serotonin transporter system in PTSD. Other baseline studies of platelet serotonin concentration and 5-HT1A receptor expression could not detect any differences between PTSD patients and controls (Bonne et al., 2005; Pivac et al., 2006). In pharmacological challenge studies, PTSD has been associated with a blunted prolactin response to d-fenfluramine (Davis et al., 1997), but not to buspirone (Dinan et al., 1990). Administration of the predominantly 5-HT2 and 5-HT1C agonist, m-chloro-phenylpiperazine (mCPP), has however, been shown to elicit panic attacks in 31% of PTSD patients (Southwick et al., 1997).

Although there have been a limited number of studies regarding serotonergic function in PTSD, there exists a large body of indirect evidence for a role of this neurotransmitter in
the pathophysiology of the disorder. For example, PTSD often presents with symptoms of aggression, impulsivity, depression and suicidality, processes all believed to be mediated by 5-HT dysregulation (Vermetten and Bremner, 2002b). Perhaps the most compelling evidence for serotonergic involvement in PTSD, comes from the efficacy of SSRIs like fluoxetine in large, randomised, double-blind, controlled trials (Connor et al., 1999; Martenyi et al., 2002; Van der Kolk et al., 1994)

**Clinical Relevance**

Given the role of serotonin in regulating the endocrine stress response, anxiogenesis and anxiolysis, and the efficacy of SSRIs in PTSD, dysfunction of serotonergic neurotransmission undoubtedly contribute to the pathophysiology of PTSD. For example, increased stimulation of 5-HT$_2$ receptors in the amygdala and hippocampus or decreased stimulation of 5-HT$_1$ receptors in the hippocampus may lead to increased anxiety (Vermetten and Bremner, 2002b). Furthermore, 5-HT is involved in the stress response, with serotonergic systems contributing to either facilitation or inhibition of basal and stress-induced glucocorticoid secretion (Lowry, 2002). Dysregulation of serotonergic neurotransmission may therefore contribute to the abnormal neuroendocrine stress response associated with PTSD. Finally, 5-HT is a counter-regulatory neurotransmitter in the LC that dampens noradrenergic firing (Newport and Nemeroff, 2000). Therefore, reduction in serotonergic neurotransmission in PTSD may contribute to abnormal LC/noradrenergic system functioning. However, the extent of this involvement in PTSD and its interaction with other systems remains to be fully elucidated.

**2.5.5.4 The Dopaminergic System**

Anatomically, there are four major dopaminergic pathways in the brain, namely the mesolimbic-, mesocortical-, nigrostriatal-, and tuberoinfundibular pathways. The mesolimbic pathway mediates dopaminergic projections from nuclei in the ventral tegmental area in the midbrain to the nucleus accumbens in the limbic system and is believed to be involved in processes of reward and hedonia (Kienast and Heinz, 2006). The mesocortical pathway connects the ventral tegmental area to the cortex, particularly the frontal lobes, is essential for normal cognitive functioning of the prefrontal cortex and
is associated with motivation, reward and emotional response. The nigrostriatal pathway
transmits DA from the substantia nigra to the striatum and is particularly involved in
motor control, as part of the basal ganglia motor loop. Finally, the tuberoinfundibular
pathway refers to a population of dopaminergic neurons in the arcuate nucleus of the
mediobasal hypothalamus that project to the median eminence of the pituitary. This
pathway is involved in neuroendocrine regulation and prolactin (Kienast and Heinz, 2006;
Wikipedia, the free encyclopaedia, 2006).

The various actions of DA are mediated by specific receptors that have been classified as
either D₁-like (D₁A-D and D₅) or D₂-like (D₂, D₃ and D₄) based on pharmacology and
second messenger system coupling (Jaber et al., 1996). The relative concentration of D₁-
like receptors is higher compared to D₂ receptors in the prefrontal cortex, whereas the
concentration of D₂-like receptors is higher in the caudate nucleus, putamen and nucleus
accumbens (Kienast and Heinz, 2006).

Preclinical studies show that stress may have a negative impact on the normal physiology
of the central dopaminergic system (Pani et al., 2000). In this regard, the dopaminergic
innervation of the medial prefrontal cortex seems to be particularly vulnerable to even
mild and brief stress, compared to mesolimbic and nigrostriatal systems, which are
typically only affected by higher levels of stress (Bremner et al., 1999). Stress-induced
increases of DA release are susceptible to modulation by several other chemical
mediators of the stress response including GABA, EAAs, 5-HT, substance P, opioids,
CRH, and NE (Bale, 2005; Vermetten and Bremner, 2002a).

In summary, the dopaminergic system is important for hedonic impact and reward
learning. In a broader sense, DA is also essential for functions such as reactivity to
changes in environmental conditions, general emotional responses, and selective
information processing. Thus, stress may directly influence several behaviours mediated
by DA and subsequently, lead to failure to cope with the external world (Pani et al.,
2000). It is therefore not surprising that dopaminergic dysfunction has been suggested to
contribute to the development of PTSD (Deutch and Young, 1995).
Dopaminergic Alterations in PTSD

Relatively few studies of dopaminergic function in PTSD have been published and most of these have reported significantly higher urinary levels of DA and its major metabolite (homovanillic acid) in PTSD patients compared to controls (De Bellis et al., 1999; Glover et al., 2003; Lemieux and Coe, 1995; Spivak et al., 1999; Yehuda et al., 1992). These results are in accordance with a preliminary report of elevated plasma DA levels in PTSD subjects compared to controls (Hammer and Diamond, 1993). Peripheral measurement of DA is, however, a poor indication of changes in central dopaminergic systems (Deutch and Young, 1995) and no studies have directly examined dopaminergic brain systems in PTSD. Some (Sher et al., 2005), but not all (Geracioti et al., 2001) indirect studies of central dopaminergic activity suggest that PTSD is associated with increased CSF homovanillic acid levels.

In summary, clinical studies to date, although preliminary, suggest increased dopamine release and metabolism in PTSD.

Clinical Relevance

As mentioned earlier, the dopaminergic system plays an important role in processes such as reward, hedonic impact, and emotional responses, and subsequently, determines the ability to cope with the external world (Pani et al., 2000). Stress induces alterations in dopaminergic systems, with the medial prefrontal cortex dopaminergic innervation being particularly sensitive to stress (Bremner et al., 1999). The prefrontal cortex is believed to be important in selective information processing and attention, working memory and extinction of conditioned fear (Fuster, 2001) and a critical range of DA turnover is necessary to maintain optimal functioning of this system (Vermetten and Bremner, 2002a). Thus, dysfunctional central dopaminergic activity may cause cognitive impairment, reduced extinction of conditioned fear, coping deficits, and abnormal motivational and reward mechanisms, thereby contributing to re-experiencing, hyperarousal and avoidance symptoms in PTSD (Charney, 2004; Deutch and Young, 1995).
2.5.5.5 Endogenous Benzodiazepines

Endogenous benzodiazepines are found in the periphery, as well as throughout the brain and play an important role in the stress response and anxiety (Vermetten and Bremner, 2002a). Their mechanism of action involves the potentiation and prolonging of synaptic actions of the inhibitory neurotransmitter GABA, via interaction with distinct binding sites on the GABA<sub>A</sub> receptor complex (Charney et al., 2001).

Preclinical studies demonstrate a stress-induced decrease in benzodiazepine receptor binding in the cerebral cortex, frontal cortex, hippocampus, and hypothalamus, which is associated with alterations in memory (Avital et al., 2001; Drugan et al., 1986; Drugan et al., 1986; Weizman et al., 1989). Initial clinical studies also show decreased platelet peripheral benzodiazepine receptor density in PTSD patients compared to controls (Gavish et al., 1996), as well as decreased (Bremner et al., 2000) or unchanged (Fujita et al., 2004) iomazenil distribution volumes in the in the prefrontal cortex of PTSD patients compared to controls.

In summary, the results from preclinical stress studies and the observation that anxiety in PTSD often respond to benzodiazepine administration, suggest dysregulation of endogenous benzodiazepine systems in PTSD. Some, but not all, clinical studies support decreased benzodiazepine receptors density and/or affinity in PTSD, and it has been suggested that this phenomenon may represent a genetic risk factor for the development of the disorder (Charney, 2004).

2.5.5.6 Other Stress Systems

Preclinical and clinical studies suggest the involvement of several other brain systems in stress and PTSD, including the opioid-, NPY-, somatostatin-, endogenous benzodiazepine-, thyroid- and neuro-immune systems (Vermetten and Bremner, 2002a) and are noted here for the sake of completion.
3.1 Introduction

Traditional studies on the pathophysiology of stress-related disorders such as post-traumatic stress disorder (PTSD) and depression have focused on changes in monoamines and their receptors. This was based on the finding that stress has notable effects on these neurotransmitter systems (Carrasco and Van de Kar, 2003) and that most of the clinically effective drugs for depression and PTSD were found to act primarily by inhibiting the re-uptake or breakdown of serotonin (5-HT) and/or norepinephrine (NE) (Schloss and Henn, 2004).

The monoamines and many other neurotransmitter systems result in the alteration of various receptor-coupled intracellular signal transduction pathways (Duman, 1995) and actions on synaptic NE and/or 5-HT may not be the primary or only mechanism by which antidepressants exert their therapeutic effects. While the inhibition of neurotransmitter uptake or breakdown occurs within hours after the first dose, the clinical antidepressant effects are only observed after chronic administration (Faure et al., 2006; Robinson, 2003). This time-course of antidepressant efficacy has led to the suggestion that some effect downstream of increased synaptic neurotransmitter level may ultimately be responsible for their therapeutic efficacy (Zarate et al., 2003). Cellular changes that take time to emerge such as alterations in post-receptor pathways, gene expression and neuroplasticity may therefore play an essential role in the clinical effects of antidepressants (Gould and Manji, 2002). In accordance with this hypothesis, studies have since shown that antidepressants do affect intracellular signalling networks (reviewed in Gould and Manji, 2002; Picchini et al., 2004) and neuronal plasticity (Duman et al., 1999).
Further evidence supporting the “downstream hypothesis” of antidepressant action comes from the observation that not all clinically effective antidepressants induce increased synaptic monoamine levels. In fact, the mechanism of action of tianeptine is traditionally assumed to involve enhanced serotonin re-uptake (Kamoun et al., 1994), yet its efficacy in depression and PTSD, and its side effect profile is similar to that of classic antidepressants (Önder et al., 2005; Wagstaff et al., 2001). Lithium, a mood stabiliser and antidepressant (Bauer et al., 2000), has beneficial effects in the treatment of PTSD on symptoms such as anxiety, anger, irritability, and insomnia (Forster et al., 1995; Kitchner and Greenstein, 1985). Lithium’s mechanism of action appears to involve effects on neurotransmission (Chenu and Bourin, 2006) and multiple signalling cascades including inositol-, apoptotic- and neurotrophic pathways (Yaun et al., 2004). Thus, it can be argued that through their different initial mechanisms, clinically effective antidepressant drugs might be reaching the same common endpoint at the intracellular level, resulting in similar clinical efficacy.

Finally, evidence points to neuronal atrophy and loss of plasticity in response to stress, especially repeated or sustained stress (McEwen and Magarinos, 2001; Radley and Morrison, 2005). Moreover, it is argued that antidepressants can oppose or reverse these adverse cellular effects (Duman et al., 1999). These findings have led to the suggestion that alterations in neurogenesis, neuroplasticity and neuronal survival or resilience may underlie the pathophysiology of stress-related disorders and that therapeutically effective drugs may act by increasing or restoring abnormalities in these processes.

Studies on intracellular cascades and their interactions with stress will provide further understanding of the pathophysiology of depression and PTSD (Coyle and Duman, 2003). Furthermore, identifying dysregulation of neuronal resilience-, plasticity- and survival pathways in these disorders could provide novel targets for therapeutic intervention and ultimately lead to the development of better, faster acting drugs (Tardito et al., 2006). It is the aim of this chapter to present current knowledge on anxiety and stress as a disorder of neuronal resilience and plasticity and that PTSD, the emphasis of the current study, represents changes in these pathways.
3.2 Impairment of Neuronal Plasticity and Resilience

Neuronal plasticity or neuroplasticity is defined as the ability of the brain to adapt to a variety of internal and external stimuli in order to optimise its function (Fuchs et al., 2004) and includes alterations of long-term potentiation (LTP), dendritic function, synaptic remodelling, axonal sprouting, neurite extension, synaptogenesis, and even neurogenesis (Charney et al., 2004). Disrupted or abnormal plasticity could consequently lead to maladaptive neuronal functioning and behaviour (Duman, 2004).

3.2.1 Evidence

Evidence for impairment of neuronal plasticity and resilience in stress and stress-related disorders comes from clinical imaging and post-mortem studies, preclinical studies on the effect of stress and glucocorticoids, and studies on the effects of antidepressant treatment.

3.2.1.1 Clinical Studies

Structural brain imaging studies in PTSD have revealed reduced hippocampal (Karl et al., 2006; Kitayama et al., 2005; Smith, 2005) and amygdala volume (Karl et al., 2006), as well as reduced hippocampal neuronal integrity (Chen et al., 2006; Li et al., 2006a; Yang et al., 2006). Similarly, structural imaging studies in depression demonstrate a reduction in the volume of the hippocampus (Campbell et al., 2004; Videbech and Ravnkilde, 2004), as well as the amygdala, although less consistently (Campbell and MacQueen, 2006). In addition, decreased volumes have also been reported for the orbital and medial prefrontal cortex, basal ganglia and cerebellum in depressed subjects (Beyer and Krishnan, 2002; Campbell and MacQueen, 2006).

Functional imaging studies in PTSD indicate diminished hippocampal activity during retrieval of memories of abuse or a declarative memory task (Bremner et al., 1999a; Bremner et al., 2003a; Bremner et al., 2003b). The PTSD prefrontal cortex also demonstrates impaired responsiveness to traumatic reminders or fearful stimuli (Bremner
et al., 1999c; Bremner et al., 1999a; Lanius et al., 2001; Lindauer et al., 2004a; Shin et al., 1999; Shin et al., 2004a; Shin et al., 2005). In contrast, patients with PTSD exhibit greater amygdala responses when exposed to reminders of traumatic events or fearful stimuli (Hendler et al., 2003; Pissiota et al., 2002; Protopopescu et al., 2005; Shin et al., 1997; Shin et al., 2005). With regard to mood disorders, functional imaging studies have demonstrated a consistently increased activity within limbic regions, with decreased activity in dorsal and ventral prefrontal regions (Drevets, 2000; Haldane and Frangou, 2006).

Postmortem brain studies in mood disorders also demonstrate reduced volume, thickness or size of cortical areas and several subcortical nuclei (Bielau et al., 2005; Bouras et al., 2001; Bremner et al., 2002; Rajkowska et al., 1999) and a reduction in neuronal size or density of especially cortical regions and the CA2 region of the hippocampus (Rajkowska, 2002). Although one study has detected significant apoptosis in selected hippocampal subregions of the majority of depression subjects, massive cell loss is not believed to contribute significantly to hippocampal volume loss in depression (Lucassen et al., 2001a). Finally, glial cell number/density and size also seem to be reduced in the amygdala and several cortical regions (Cotter et al., 2001; Ralkowska, 2002). Although, post-mortem studies in PTSD are limited (Krystal and Duman, 2004; Osuch et al., 2004), lower locus coeruleus neuronal counts have been noted (Bracha et al., 2005).

Controversy remains whether the above brain volume changes occur as a result of the disease, are genetically pre-determined, or are caused by developmental changes (Sala et al., 2004; Spedding et al., 2003). However, glial changes may contribute to neuronal pathology and stress-related disorders (Cotter et al., 2001; Manji et al., 2003) since they play a major role in regulating energy metabolism and glutamate and neurotrophin release (Field and Stevens-Graham, 2002; Villegas et al., 2003).

### 3.2.1.2 Preclinical Studies

Studies in rodents and tree shrews show that repeated or prolonged restraint or psychosocial stress leads to alterations of neuroplasticity not only in the hippocampus, but also in the prefrontal cortex and amygdala (Fuchs and Flüge, 2003; McEwen, 2004).
Chronic or repeated stress leads to a decrease in the number and length of apical dendrites in the CA3 region of the hippocampus (Magarinos et al., 1996; Watanabe et al., 1992b), which seems to be reversible when animals are allowed to recover from stress (Conrad et al., 1999; Sousa et al., 2000). Long-term or sustained exposure to stress, however, can result in true loss of hippocampal CA3 neurons (Sapolsky, 2000). Chronic stress also impairs neurogenesis in the dentate gyrus (Gould et al., 1997; Gould et al., 1998) and decreases the number and cell body volume of astroglia (Czeh et al., 2006), with little effect on apoptosis (Lucassen et al., 2001b). In the hippocampal CA1 and dentate gyrus, profound alterations in mossy fibre terminal morphology and significant synapse loss in response to stress have also been reported (Sousa et al., 2000). Finally, stress may make hippocampal cells more vulnerable to other types of insults (Manji and Duman, 2001).

Like the hippocampus, pyramidal neurones in the prefrontal cortex show apical dendrite atrophy in response to chronic or mild, repeated restraint stress (Brown et al., 2005; Cook and Wellman, 2004; Radley et al., 2004; Radley et al., 2006).

In contrast to the atrophy observed in the hippocampus and prefrontal cortex, stress appears to induce hypertrophy in the basolateral amygdala. Chronic immobilisation stress in rats induces an increase in dendritic growth and density of spiny neurones, which persists even after recovery (Bennur et al., 2007; Vyas et al., 2002; Vyas et al., 2004; Vyas et al., 2006).

### 3.2.1.3 Effects of Antidepressants

In a clinical study of PTSD patients, chronic treatment with the antidepressant paroxetine reversed hippocampal volume reduction, an effect that was accompanied by significant improvement of memory deficits (Vermetten et al., 2003). In a clinical study of depression however, the antidepressant induced improvement in memory and depression symptoms was not accompanied by changes in hippocampal volume (Vythilingam et al., 2004).

In pre-clinical studies, various classes of antidepressants have been shown to give opposite effects to those of stress on hippocampal neurogenesis and neuronal
morphology, for example, increased hippocampal neurogenesis and cell proliferation (Encinas et al., 2006; Larsen et al., 2007). Long-term antidepressant treatment also prevents or reverses stress-induced atrophy and decreases in cell proliferation, neurogenesis, volume and astroglial plasticity in the hippocampus (Czeh et al., 2001; Czeh et al., 2006; Jayatissa et al., 2006; Li et al., 2004b; Malberg and Duman, 2003; Xu et al., 2006a). Significantly, some of these effects appear essential for the actions of antidepressants in behavioural models of depression (Santarelli et al., 2003).

The molecular mechanism by which antidepressants achieve their positive modulatory effects on the hippocampus have been proposed to involve the increased expression of prosurvival factors such as brain derived neurotrophic factor (BDNF), cyclic AMP response element binding protein (CREB), protein kinase B (PKB or Akt) and BCL-2 (Chen and Russo-Neustadt, 2005; Larsen et al., 2007; Sairanen et al., 2007; Tardito et al., 2006; Xu et al., 2003), as well as the decreased expression or activity of possible anti-survival factors such as nitric oxide synthase (NOS) and glycogen synthase kinase (GSK)-3β (Li et al., 2004c; Li et al., 2006c; Luo and Tan, 2001; Wegener et al., 2003).

3.2.2 Underlying Cellular Mechanisms in Stress

Mechanisms for the underlying harmful effects of stress on neuroplasticity and hippocampal volume are believed to involve glucocorticoids, glutamate excitotoxicity and reduced neurotrophic factor release.

3.2.2.1 Glucocorticoids

Clinical studies of stress-related disorders often report alterations in hypothalamic-pituitary-adrenal (HPA) axis activity, with depression and PTSD being associated with increased and decreased peripheral cortisol, respectively (reviewed in De Kloet et al., 2006 and Gillespie and Nemeroff, 2005). It should be mentioned however, that there have been some reports of elevated basal cortisol levels in PTSD (Inslicht et al., 2006; et al., 1998), and most stress-challenge tests have found increased cortisol levels (Bremner et al., 2003c; Elzinga et al., 2003) and enhanced target tissue sensitivity to glucocorticoids (Yehuda et al., 2004). Cushing syndrome, which is characterized by pathological
oversecretion of glucocorticoids, is also associated with hippocampal atrophy, providing further support for a role of glucocorticoids in brain abnormalities in stress-related disorders (Sapolsky, 2000; Starkman et al., 1999). Finally, clinically effective antidepressants have been shown to reduce HPA axis responsiveness in depression (Nikisch et al., 2005) and reduce stress-induced cortisol overactivity in PTSD (Vermetten et al., 2006).

In pre-clinical studies, chronic glucocorticoid administration replicates the effects of stress on neuroplasticity, whereas administration of an adrenal steroid synthesis blocker prevents this (Magarinos and McEwen, 1995; Cerqueira et al., 2005; Wellman, 2001). While the amygdala and prefrontal cortex also contain adrenal steroid receptors, the role of glucocorticoids in the stress-induced alterations of these brain regions remains speculative (McEwen, 2005).

Glucocorticoids are believed to damage the hippocampus by disrupting cellular metabolism (Kadekaro et al., 1988; Virgin et al., 1991), causing accumulation of synaptic glutamate (Leza et al., 1998; Virgin et al., 1991), increasing the free cytosolic Ca^{2+} concentrations (Elliott and Sapolsky, 1993), worsening oxygen radical accumulation during insults (McIntosh et al., 1998) and inhibiting neuronal defences (reviewed in Sapolsky, 2000).

However, the absence of glucocorticoids also has negative impacts on the hippocampus, as adrenalectomy evokes apoptosis and impairs synaptic transmission (Sloviter et al., 1993; Stienstra et al., 1998). In contrast, another study has shown that acute adrenalectomy leads to increased hippocampal neurogenesis (Brunson et al., 2005). Evidence suggests that mineralocorticoid receptors (MRs), which are activated at low levels of glucocorticoids, are selectively involved in stress-induced hippocampal changes. In this regard, genetic disruption of MRs but not glucocorticoid receptors (GRs) impairs neurogenesis and granule cell degeneration in the adult mouse hippocampus (Gass et al., 2000).
**Glutamate Excitotoxicity**

Drugs that reduce \( N \)-methyl-D-aspartate (NMDA) receptor function such as D-cycloserine and ketamine have antidepressant activity (Crane, 1959; Zarate et al., 2006), while and D-cycloserine also has some efficacy in the treatment of PTSD symptoms (Heresco-Levy et al., 2002). Data from clinical glutamate receptor studies, magnetic resonance spectroscopy (MRS) studies and cerebrospinal fluid (CSF) or peripheral glutamate/glutamine studies also provide some support for the involvement of glutamate in stress-related disorders (reviewed in Manji et al., 2003; Sanacora et al., 2003).

Chronic glucocorticoid treatment has also been found to up-regulate the NMDA receptor system (Weiland et al., 1997). Administration of anti-glutamatergic drugs block the stress-induced atrophy of hippocampal CA3 neurones (Magarinos and McEwen, 1995; Watanabe et al., 1992a), and the vulnerability of hippocampal neurones following glucocorticoid exposure are also dependent on NMDA receptor activation (Armanini et al., 1990).

The mechanism by which glutamate is believed to mediate the harmful effects of stress is suggested to involve sustained activation of NMDA receptors, \( \text{Ca}^{2+} \) mobilisation and \( \text{Ca}^{2+} \)-dependent cytoskeletal damage, oxidative damage and apoptosis following the generation of free radicals (Madrigal et al., 2006; Sapolsky, 2000). Glutamate may also induce the release of the pro-inflammatory cytokine tumour necrosis factor (TNF)-\( \alpha \), leading to activation of the transcription factor nuclear factor kappa beta (NF\( \kappa \)B) and induction of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase 2 (COX2) (Madrigal et al., 2004; Madrigal et al., 2006).

**Neurotrophic Factors**

The nerve growth factor (NGF) family, consisting of the neurotrophins BDNF, neurotrophin-3, neurotrophin-4/5 and neurotrophin-6 (Lang et al., 2004) mediates neuronal survival and modulates neuroplasticity, synaptic activity and neurotransmitter synthesis in the adult central nervous system (Poo, 2001). Neurotrophins promote cell survival via the inhibition of apoptotic mechanisms, rather than by inducing cell survival.
pathways (Charney et al., 2004; Rich, 1992) so that a pathological alteration of neurotrophin action may lead to impaired neuroplasticity, as well as neuronal death (Huang and Reichardt, 2001; Lang et al., 2004).

Clinical and post-mortem studies provide some support for a role of BDNF in stress-related disorders. Serum BDNF levels have been found to be significantly lower in untreated, depressed patients than treated, depressed patients and control groups (Shirnizu et al., 2003). Increased BDNF expression has also been found in post-mortem hippocampus of antidepressant-treated depressive, bipolar, and schizophrenic subjects compared with untreated subjects (Chen et al., 2001).

Some (Gronli et al., 2006; Kozlovsky et al., 2007; Murakami et al., 2005; Pizarro et al., 2004; Rasmusson et al., 2002), but not all (Kuroda and McEwen, 1998) pre-clinical studies have shown that stress negatively regulates BDNF in the hippocampus (reviewed in Duman and Monteggia, 2006). An inhibiting effect for stress on the expression of other neurotrophic factors such as NGF, neurotrophin-3 and vascular endothelial growth factor has also been demonstrated (Alfonso et al., 2006; Heine et al., 2005; Ueyama et al., 1997). There have also been reports where stress failed to affect hippocampal neurotrophic factor levels (Kuroda and McEwen, 1998). In addition, some studies even report a stress-induced increase in hippocampal neurotrophic factor levels, possibly reflecting a compensatory mechanism (Faure et al., 2006). Despite this diversity in results, chronic corticosterone administration has been shown to mimic the stress-induced decrease in hippocampal neurotrophic factor levels, while adrenalectomy results in decrease in BDNF mRNA (Chao et al., 1998; Jacobsen and Mork, 2006). Moreover, the regulation of neurotrophic factors by glucocorticoids is dependent on receptor subtype (GR, MR), as well as brain region (Chao et al., 1998; Hansson et al., 2000).

In conclusion, stress-induced and glucocorticoid associated alterations in neurotrophic factor levels in general, and BDNF in particular, may contribute to neuronal atrophy/death and reduction of hippocampal volume.
3.3 Signalling in Neuronal Plasticity and Resilience

Signal transduction involves the transmission of extracellular signals to intracellular responses. In general, the binding of a ligand to its receptor causes the receptor to undergo a conformational change, triggering a signalling cascade involving a network of proteins that act on one another to generate a response (Signal transduction, 2005). Phosphorylation by kinases and dephosphorylation by phosphatases play very important roles during these signal transduction cascades. It is important to keep in mind that signalling pathway function together to form intricate, dynamic networks by means of crosstalk or cross-signalling (Dumont et al., 2001), while at the same time, adequate signalling specificity is essential to obtain the right response at the right time and place (Schenk and Snaar-Jagalska, 1999).

The purpose of this section is to provide an overview of extracellular receptors involved in signalling, followed by a discussion of some of the pathways believed to play a role in neuronal plasticity and resilience. Thereafter, specific signalling molecules that form part of these pathways will be discussed in terms of their regulation, targets and involvement in stress and stress-related disorders.

3.3.1 Cell Surface Receptors

Neurotransmitters and neuropeptides reported to be involved in neuronal plasticity and resilience such as 5-HT, NE, dopamine (DA), glutamate and BDNF activate different types of cell surface receptors include G protein-coupled receptors (GPCRs), receptor tyrosine kinase receptors (RTKs) and ligand-gated ion channels (Bury and Cross, 2003), to transduce extracellular signals into the interior of the cell.

The G protein family is divided into four major classes, namely $G_\alpha$, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ and each class has a general mechanism of signal transduction. Stimulation of the $G_\alpha$ class activates adenylate cyclase (AC), whereas stimulation of the $G_i$ class inhibits this enzyme.
Stimulation of the $G_q$ class activates phospholipase C (PLC), whereas the $G_{12}$ class has been implicated in the regulation of small GTP binding proteins (Hur and Kim, 2002).

The RTKs are activated by neurotrophins (BDNF, NGF) and several growth factors (Heldin, 1995). RTKs consist of an extracellular ligand binding domain connected to a cytoplasmic domain, which can be autophosphorylated or phosphorylated by protein kinases (Schlessinger, 2000). This phosphorylation of tyrosine residues results in full kinase activation (Friedman and Greene, 1999) and signalling via different intracellular signalling cascades, including the mitogen activated protein kinase (MAPK)-, phospholipase C (PLC)-, and phosphoinositide-3 kinase-protein kinase B (PI3K-Akt) pathways (Schlessinger, 2000).

Ion channel receptors can be either voltage- or ligand-gated. Ligand-gated ion channels are transmembrane receptors that form a binding site for the ligand and an ion-conducting pore (Hogg et al., 2005), and include the cys-loop receptors (nicotonic-, 5-HT$_3$-, GABA$_A$-, GABA$_C$ receptors), the ATP gated channels, and the ionotropic glutamate receptors (NMDA, AMPA and kainate) (Hogg et al., 2005). If opened these channel receptors allows selective flux of cations or anions, depending on the receptor type (Leite and Cascio, 2001), leading to changes in membrane polarization and activation of intracellular signalling pathways (Du et al., 2006).

### 3.3.2 Pathways in Neuronal Plasticity, Resilience and Survival

Some of the pathways believed to be involved in neuronal plasticity, resilience and survival are depicted in figure 3.1 and include cyclic adenosine monophosphate-response element-binding protein (cAMP-CREB), PLC-, PI3K- and extracellular regulated (ERK) pathways, NOS signalling and apoptotic signalling.
Figure 3.1 G protein-coupled receptor-, tyrosine kinase receptor- and Wnt- mediated signalling pathways involved in neuronal plasticity, resilience and survival (Picchini et al., 2004). See text for detailed descriptions of individual pathways.

3.3.2.1 The cAMP-CREB Pathway

The cAMP-CREB pathway is well characterised and known to be involved in the pathophysiology and treatment of stress-related disorders (Gould and Manji, 2002).

This signalling system is controlled by the action of neurotransmitters, neuromodulators or hormones on GCPRs, which stimulate or inhibit the enzyme AC. Once activated, AC converts adenosine triphosphate (ATP) into the second messenger cAMP (Baillie et al.,
2005), which is negatively controlled by a family of phosphodiesterase (PDE) enzymes, resulting in the formation of the inactive 5'-monophosphate (Essayan, 2001). The main effect of cAMP is the activation of protein kinase A (PKA), resulting in the phosphorylation of numerous proteins including ion channels, cytoskeletal elements and other enzymes such as GSK-3 and dopamine-and-cAMP-regulated phosphoprotein (DARPP-32) (Daniel et al., 1998; Gould & Manji, 2002; Jope and Johnson, 2004). PKA can also translocate into the nucleus, where it targets transcription factors such as CREB (as shown in figure 3.1), cAMP response element modulator (CREM), activating transcription factor-1 (ATF-1), NFκB and nuclear receptors (Daniel et al., 1998).

### 3.3.2.2 The PLC Pathway

The PLC pathway is a well characterised signal transduction pathway that is activated in response to many neurotransmitters, neuropeptides and neurohormones. Eleven mammalian PLCs have been identified (PLCβ1-4, PLCγ1-2, PLCδ1-4 and PLCε) which are activated by different mechanisms (Parker and Murray-Rust, 2004; Rhee and Bae, 1997), but all catalyse the conversion of phosphatidylinositol (PtdIns)(4,5)P2 (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG) (Hur and Kim, 2000; Pacheco and Jope, 1996). These products serve as second messengers in two separate transduction cascades. IP3 binds to receptors and mediates the release of Ca2+ from intracellular stores (Duman, 1995) and subsequently, the activation of regulatory enzymes including Ca2+/calmodulin-dependent kinases (CaMKs) and some isoforms of protein kinase C (PKC). DAG also plays a role in PKC activation.

#### CaMKs

An increase in intracellular Ca2+ leads a conformational change in calmodulin and the resulting Ca2+/calmodulin complex activates CaMKs. CaMKs are classified as either dedicated CaMKs, which have strict substrate specificities, or multifunctional CaMKs, which can phosphorylate multiple proteins including ion channels, signalling molecules, factors regulation apoptosis, scaffolding proteins and transcription factors (Ishida et al., 2003; Reith, 2000; reviewed in Soderling, 2000).
PKC

The PKC family consists of conventional isoforms (PKCa, PKCb and PKCy) that are DAG and Ca\(^{2+}\) responsive, novel isoforms (PKCD, PKCe, PKC\(\gamma\),PKCF) that respond to DAG, but are Ca\(^{2+}\) insensitive and atypical isoforms that are not regulated by DAG or Ca\(^{2+}\) (Parker and Murray-Rust, 2004). Once activated, PKC phosphorylates substrates that include transcription factors (CREB; NF-\(\kappa\)B), cytoskeletal components (MARCKS, GAP34), enzymes (NOS, GSK-3\(\beta\)), ion channels, transporters, receptors, trafficking proteins, and adhesion proteins (Brose et al., 2004; Larsson, 2006; Riccio et al., 1996; Schnick and Snaar-Jagalska, 1999). In addition, PKC is known to crosstalk with several other signalling cascades, notably the MAPK/ERK-, NF-\(\kappa\)B- and apoptotic signalling pathways (Yang and Kazanietz, 2003).

3.3.2.3 PI3K Pathway

The PI3Ks are into 3 distinct classes (I, II and III) according to their molecular structure, mode of activation and substrate specificity (Oudit et al., 2004), of which class I is primarily responsible for the production of position 3 phosphoinositides coupled to extracellular stimuli of cell surface receptors, including RTKs and GPCRs (Wymann et al., 2003; Wymann and Marone, 2005). Once activated, class I PI3Ks predominantly phosphorylate PtdIns(4,5)P\(_2\) to form PtdIns(3,4,5)P\(_3\). By binding to specific protein domains, PtdIns(3,4,5)P\(_3\) associated with Akt and PI3K dependent protein kinase 1 (PDK1) at the cell membrane to enable the phosphorylation and activation of Akt (as shown in figure 3.1) (Kaplan and Miller, 2000; Oudit et al., 2004; Patapoutian and Reichardt, 2001). Other targets of PI3K include inhibitors of apoptosis (IAPs), some PKC isoforms, and p70 S6 kinase (Duronio et al., 1998).

3.3.2.4 ERK Pathway

The MAPK superfamily consists of the ERKs, the c-Jun N-terminal/stress activated protein kinases (JNKs/SAPKs) and the p38 kinases (Pearson et al., 2001). All MAPK pathways consist of a three step sequential kinase cascade, namely a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK that allows for signal...
amplification and cross-talk with other signalling pathways (Cobb, 1999; Pearson et al., 2001).

ERK1 and ERK2 (ERK1/2) are dually phosphorylated and activated by a pair of MAPKKs known as MAP/ERK kinase (MEK)1 and MEK2. The MEKs in turn, are phosphorylated and activated by a MAPKKK or MEKK known as Raf (Cowan and Storey, 2003). The ERK pathway can be activated by RTKs (as shown in figure 3.1), as well as via second messengers such as Ca^{2+}, cAMP and DAG (Grewal et al., 1999; Liebmann, 2001). Indeed, GPCR signalling to ERK cascades has been studied extensively and may be mediated by different classes of G proteins including and may involve direct phosphorylation and activation or inhibition of a Raf family member via PKC, PKA or Akt, or “transactivation” of RTKs via non-receptor tyrosine kinases (Finkbeiner and Greenberg, 1996; Liebmann, 2001; Sweatt, 2004).

**3.3.2.5 NO Signalling**

Nitric oxide (NO) is a highly diffusible and reactive molecule and unique second messenger in the brain, proposed to be involved in a wide range of physiological and pathological events, including neurogenesis, neuroplasticity, neurotransmission and neuronal apoptosis (Cárdenas et al., 2005; Contestabile and Ciani, 2004; Prast and Philippu, 2001).

**NO Synthesis**

NO is synthesised by three NOS isoforms, namely neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Duncan and Heales, 2005). Another isoform, known as mitochondrial NOS (mNOS) has also been identified (Elfering et al., 2002).

The binding of Ca^{2+}-calmodulin regulates the catalytic activity of nNOS and once activated, the enzyme converts l-arginine to citrulline, with NO as a by-product (Alderton et al., 2001). Because the activity of nNOS is critically dependent on Ca^{2+}, the enzyme can be activated by extracellular surface receptors coupled to Ca^{2+} second messenger systems (Hu and El-Fakahany, 1996), such as some GPCRs and NMDA, AMPA and
kainate receptors (figure 3.2) (Duman, 1995; Hu and El-Fakahany, 1996). Finally, an increase in intracellular \( \text{Ca}^{2+} \) by other mechanisms may also activate nNOS (Hu and El-Fakahany, 1996).

Apart from the critical stimulus allowing \( \text{Ca}^{2+} \) influx, nNOS may also be regulated by expression, alternative mRNA splicing, post-translational modifications (phosphorylation) and protein-protein interactions, for example with protein inhibitor of NOS (PIN), PDS-95 and with carboxy-terminal PDZ ligand of NOS (CAPON) (Alderton et al., 2001; Hu and El-Fakahany, 1996; Ledo et al., 2004; Mcleod et al., 2001). Although nNOS was originally believed to be constitutively expressed, evidence indicates that its expression levels are dynamically regulated in response to extracellular stimuli (Dawson et al., 1998). In this regard, glucocorticoids have been shown to inhibit the expression of nNOS in the hippocampus (Lopez-Figueroa et al., 1998). Finally, NO itself is capable of regulating nNOS activity via mechanisms involving S-nitrosylation of nNOS and inhibition of NMDA receptor activity (Alderton et al., 2001; Hu and El-Fakahany, 1996; Lei et al., 1992; Manzoni et al., 1992).

*Figure 3.2 nNOS signalling (Ledo et al., 2004). See text for details.*
In contrast to nNOS, iNOS is independent of Ca\(^{2+}\) due to a tight interaction of Ca\(^{2+}\) with calmodulin (Guix et al., 2005). In the brain, this NOS subtype is not expressed constitutively, but may be induced in glial cells at the transcriptional level by inflammatory stimuli. Upon induction, iNOS is expressed for days and produces NO in concentrations up to 1000 times those produced by nNOS (Pannu and Singh, 2006). The expression of iNOS can be induced by inflammatory cytokines such as interferon (IFN)-\(\lambda\) and interleukin (IL)-1\(\beta\), and the subsequent activation on transcription factors such as NF\(\kappa\)B, interferon regulatory factor-1 (IRF-1), CREB, CCAAT/enhancer-binding protein (C/EBP) and the signal transducers and activators of transcription (STAT) family (Duncan and Heales, 2005; Guix et al., 2005). Intracellular signalling proteins may also control iNOS expression, including PKC, tyrosine kinases, MAPKs and tyrosine kinase phosphatases (Guix et al., 2005). Other regulatory mechanisms of iNOS also include alternative mRNA splicing and protein-protein interactions (Alderton et al., 2001; Duncan and Heales, 2005).

**NO Signalling**

NO signalling pathways can be classified as soluble guanylate cyclase (sGC) dependent or -independent. NO induces a conformational change in sGC, leading to its activation and the conversion of guanosine-5'-triphosphate (GTP) to cyclic guanosine-3',5'-monophosphate (cGMP) (Guix et al., 2005). As a second messenger, cGMP is capable of modulating a multitude of cellular enzymes and proteins such as cyclic-nucleotide-gated ion channels, PDEs, and protein kinases (PKGs) (Blaise et al., 2005).

NO is a free radical, and as such, undergoes various chemical reactions to form nitrites (NO\(_2^-\)), nitrates (NO\(_3^-\)) and peroxynitrites (ONOO\(^-\)). Highly reactive intermediate products like nitrite radical (\(\cdot\text{NO}_2\)) and hydroxyl radical (\(\cdot\text{OH}\)) are also formed (Ledo et al., 2004). sGC-independent actions of NO and some of its related species involve the modification of proteins through direct chemical reactions with multiple targets, including protein nitrotyrosination and nitrosylation reactions (Guix et al., 2005). These reactions may alter the normal activity of target proteins, such as transcription factors, ion channels, nucleic acids, G-proteins, protein kinases, protein phosphatases, caspases and structural proteins and may therefore have important biological implications (Ledo et al., 2004).
**3.3.2.6 Apoptosis Signalling**

Apoptosis or programmed cell death refers to a mechanism of controlled cellular self-destruction, involving characteristic morphological changes, such as cytoplasmic shrinkage and the absence of an inflammatory response. Necrosis, on the other hand, is uncontrolled cell death and is characterised by swelling of cell organelles and a clear inflammatory response (Lossi et al., 2005).

In the developing brain, apoptosis is a vital physiological process that controls the number of neurones and glia (Lossi and Merighi, 2003). However, apoptosis also takes place in the mature central nervous system and may be associated with the loss of neurones in neurodegenerative disorders (Honig and Rosenberg, 2000). Neuronal apoptosis can be induced by neurotrophic factor withdrawal (mainly in the developing nervous system), excessive Ca\(^{2+}\) influx, glutamate excitotoxicity, oxidative stress and pro-inflammatory cytokines (Kam and Ferch, 2000), all of which have been implicated in stress. The involvement of apoptosis in the pathophysiology and treatment of stress-related disorders such as depression and PTSD, although controversial (Lucassen et al., 2001a), has been suggested (Lucassen et al., 2006; Zhang et al., 2006b).

Two major apoptotic signalling pathways have been identified, namely the extrinsic and intrinsic pathways. These two death cascades can occur independently of one another, or converge at the mitochondria (figure 3.3). Extrinsic apoptosis occurs when so-called “death-receptors” are activated by their ligands, for example, pro-inflammatory cytokines of the tumour necrosis factor (TNF) family (TNF-\(\alpha\) and Fas ligand). This leads to the activation of caspases and the induction of pro-apoptotic genes and proteins such as p53 and BAX (Benn and Woolf, 2004). Intrinsic apoptosis, on the other hand, is the result of mitochondrial damage and involves the cytoplasmic release of apoptotic molecules, including cytochrome c (cyto c) and apoptosis inducing factor (AIF). Once released, cyto c associates with apoptosis protease activating factor-1 (APAF1) and triggers caspase activation, while AIF induces neuronal apoptosis via caspase-independent mechanisms (Benn and Woolf, 2004).
Apoptosis consists of a decision phase and an execution phase. The decision phase involves genetic control of apoptosis, while the execution phase involves activation of pro-caspases and execution caspases that are ultimately responsible for inducing apoptosis via proteolysis and mitochondrial inactivation (Kam and Ferch, 2000). However, the decision to die can be reversed and damage repaired by anti-apoptotic signalling upstream, at, or downstream of mitochondria (Benn and Woolf, 2004). The anti-apoptotic BCL-2 family members BCL-2 and BCL-XL, for example inhibit apoptosis by preventing the activation of procaspases, the mitochondrial translocation of pro-apoptotic BCL-2 members BAX and BAK, and the activation of execution caspases.
Protection from apoptosis may also involve other intracellular signalling pathways, including the PI3K-Akt and ERK pathways (Kinloch et al., 1999).

### 3.3.3 Specific Messengers and Stress

#### 3.3.3.1 Akt

**Regulation**

In mammals, three isoforms of Akt (Akt1, Akt2 and Akt3) have been identified and although they are similar in structure, they are expressed differentially at both the mRNA and protein levels (Datta et al., 1999). Akts are serine/threonine kinases and phosphorylation of both the threonine (308, 309 and 305 for Akt1, 2 and 3) and serine (473, 474 and 472 for Akt1, 2 and 3) residues are required for full kinase activation (Elghazi et al., 2006).

Akt is brought to the plasma membrane after PI3K activation (section 3.3.2.3), enabling its dual phosphorylation by PDK1 and another unknown kinase (Datta et al., 1999; Oudit et al., 2004). Survival stimuli can also activate Akt by mechanisms independent of PI3K, for example via PKA and CaMK kinase (CaMKK) (Datta et al., 1999).

**Targets**

Once activated, Akt phosphorylates substrates involved in many key cellular functions including proliferation, growth, differentiation, protein synthesis, glycogen synthesis, transcription and angiogenesis and importantly, cell death and survival (Brazil and Hemmings, 2001; Elghazi et al., 2006). In this regard, Akt has been shown to be necessary for trophic factor induced survival of neurones (Brunet et al., 2001; Crowder and Freeman, 1998), as well as for protection from apoptosis induced by various insults (Brazil and Hemmings, 2001).
The mechanism whereby Akt protects against apoptosis and promotes cell survival involves its interaction with multiple substrates (Hanada et al., 2004). For example, Akt phosphorylates the serine 21 and 9 residues on GSK-3α and -β, respectively, effectively inhibiting their kinase activity and thereby potentially promoting cellular survival (Cross et al., 1995; Picchini et al., 2004). Akt may also phosphorylate and inhibit apoptotic proteins such as BAD, caspase-9 and APAF-1, as well as phosphorylate and stimulate IAPs (Brunet et al., 2001; Cardone et al., 1998; Datta et al., 1999; Zha et al., 1996). Another pro-survival mechanism of Akt is proposed to involve the induction of pro-survival gene expression via regulation of the transcription factors CREB and NFκB, both of which play an important part in neuronal survival (Brunet et al., 2001; Datta et al., 1999; Kalschmidt et al., 2005). Akt may also promote cellular survival by translocating into the nucleus and inhibiting pro-death transcription factors of the Forkhead family (Andjelkovic et al., 1997; Brunet et al., 2001). Akt also indirectly inhibits p53 (Yamaguchi et al., 2001), a transcription factor that normally induces the expression of death genes such as BAX (Schuler and Green, 2005). In summary, Akt contributes to cellular survival by inactivating apoptosis regulating proteins and genes, as well as by activating survival genes and promoting the expression of pro-survival proteins. Such multiple pathways may function as a safety mechanism, allowing for the tight control of apoptosis (Brunet et al., 2001).

Involvement in Stress

Although limited, postmortem studies indicate a possible role for Akt in stress-related disorders, such as decreased Akt activity in the ventral prefrontal cortex (Karege et al., 2007) and occipital cortex (Hsiung et al., 2003) of suicide subjects with depression compared to suicide subjects without depression. Unfortunately, no post-mortem studies regarding the role of Akt in PTSD have been published.

No studies in specific animal models of depression or PTSD have investigated Akt, although repeated immobilisation stress (sub-chronic) has been shown to increase Akt phosphorylation in the striatum, hypothalamus and hippocampus, but not in the frontal cortex (Lee et al., 2006a), suggesting that Akt phosphorylation may participate in the stress response. Furthermore, exercise, which has been shown to counteract stress related
hippocampal damage (Kiraly and Kiraly, 2005), has been shown to significantly increase hippocampal p-Akt (Chen and Russo-Neustadt, 2005).

With regard to the molecular mechanisms of antidepressants, chronic coadministration of fluoxetine and olanzapine increases in Akt phosphorylation in the prefrontal cortex, hippocampus and striatum (Maragnoli et al., 2004) and tranylcypromine enhances exercise-induced Akt phosphorylation (Chen and Russo-Neustadt, 2005). Moreover, imipramine increases the phosphorylation of Akt in differentiated neuro-2A cells (Basta-Kaim et al., 2005), while lithium, which has some benefit in the treatment of depression (Bauer et al., 2000) and PTSD (Forster et al., 1995; Kitchner and Greenstein, 1985), is neuroprotective against glutamate excitotoxicity (in vitro and in vivo) by activating the PI3K-Akt pathway (Chuang et al., 2002; Rowe and Chuang, 2004).

In summary, while the exact role of Akt in the pathophysiology and treatment of stress and stress-related disorders is unclear, limited data from postmortem and pre-clinical studies suggests some involvement.

3.3.3.2 CREB

**Regulation**

CREB and CREB-mediated gene transcription can be activated by a multitude of different physiological stimuli, including growth factors, steroid hormones, peptides, cytokines, phospholipids and environmental stress factors (Johannessen et al., 2004). Activated protein kinases such as PKA, PKG, Akt, PKC, CaMKI, II & IV, and several MAPK activating protein kinases (Daniel et al., 1998; Johannessen et al., 2004) phosphorylate CREB at serine 133 leading to the recruitment of transcriptational coactivators such as CREB binding protein (CBP) and p300 and the formation of a transcriptational complex (Bonci and Carlezon, 2005) that enables the eventual synthesis of RNA (De Sesare et al., 1999). In addition to protein kinases, protein phosphatases also regulate the phosphorylation state of CREB, either dephosphorylating it directly, or controlling its phosphorylation indirectly by controlling the enzymatic activity of CREB kinases (Johannessen et al., 2004). Additional phosphorylation sites (serine 142, 143 and 129) on
the CREB protein have also been identified (Carlezon et al., 2005; Lonze and Ginty, 2002; West et al., 2002; Wu and McMurray, 2001), while evidence also suggests the possibility of phosphorylation-independent CREB activity (Blendy, 2006). For example, NO may mediate BDNF and activity-dependent expression of CREB target genes by S-nitrosylation of nuclear proteins (Ricchio et al., 2006).

**Targets**

*In vitro* studies suggest that CREB binds to over a thousand gene promoters including those that control neurotransmission, cell structure, signal transduction, transcription and metabolism (Lonze and Ginty, 2002). However, not all of these genes are functional CREB targets and this may be dependent on cell type and brain region (Bonci and Carlezon, 2005). Examples of CREB regulated genes include genes that regulate other transcription factors, transcriptional repressors, intracellular messengers (AC), neurotransmitter synthetic enzymes (tyrosine hydroxylase), peptide transmitters (CRH), gaseous transmitters (nNOS, iNOS), growth factors and their receptors (BDNF, TrkB), apoptosis regulating proteins (BCL-2), opioid peptides and neurotransmitter subunits (GluR1) (Carlezon et al., 2005; Lonze and Ginty, 2002).

**Involvement in Stress**

CREB phosphorylation (ser 133) and activation plays an important role in neurogenesis, neuronal plasticity and neuronal survival (Duman et al., 2001; Nguyen and Woo, 2003; Walton and Dragunow, 2000) and as such, is believed to be involved in stress-related disorders (Adamiec et al., 2006; Blendy, 2006; Laifenfeld, 2005b).

Postmortem studies show that depression is associated with lower cortical CREB levels (Dowlatshahi et al., 1998) and a reduction in total CREB and p-CREB levels in the orbitofrontal and parieto-occipital cortex (Laifenfeldt et al., 2005b; Yamada et al., 2003). In contrast, other studies have found an increase in p-CREB levels in the prefrontal cortex of depressed subjects relative to controls (Laifenfeldt et al., 2005b; Odagaki et al., 2001). One of these studies found significantly increased prefrontal cortex p-CREB levels in antidepressant treated subjects (Laifenfeldt et al., 2005b), whereas the other study observed the increased prefrontal cortex p-CREB levels specifically in the antidepressant-
free subgroup (Odagaki et al., 2001). No post-mortem studies regarding the role of CREB in PTSD have been published.

Specific animal models of depression also provide support for abnormal CREB activity in stress. Congenital learned helplessness and exposure to chronic mild stress or learned helplessness stress are all associated with reduced p-CREB expression or CREB mRNA levels in the hippocampus and frontal cortex (Grønli et al., 2006; Kohen et al., 2003; Song et al., 2006; Xu et al., 2006b). Furthermore, chronic administration of imipramine and fluoxetine resulting in improved cognitive performance is associated with a significant increase in hippocampal CREB mRNA levels (Song et al., 2006). In a time dependent behavioural sensitisation model of PTSD, however, there were no differences in basal or electrified prod induced p-CREB immunoreactivity in the amygdala, locus coeruleus or hypothalamus between stressed and control rats two weeks after footshock (Bruijnzeel et al., 2001). No specific stress sensitisation model of PTSD has investigated CREB phosphorylation in the hippocampus and frontal cortex, although chronic restraint and footshock paradigms have been shown to induce a decrease in p-CREB in the frontal cortex and hippocampus in some (Alfonso et al., 2006; Trentani et al., 2002) but not all (Tretani et al., 2002) studies. Furthermore, acute predator stress (Adamec et al., 2006) and chronic social stress (Abumaria et al., 2006) significantly increases p-CREB immunoreactivity in the amygdala and CREB mRNA in the dorsal raphe nucleus, respectively.

In support of these findings, overexpression of CREB in hippocampus (Chen et al., 2001) and amygdala (after training) (Wallace et al., 2004) produces antidepressant effects, whereas amygdala CREB overexpression after training increases depressive- and anxiety-like behaviour (Wallace et al., 2004). Similarly, CREB overexpression in the nucleus accumbens has been shown to have depression-like effects (Pliakas et al., 2001). Thus, it seems that while hippocampal CREB overexpression has antidepressant-like effects, overexpression in the amygdala and nucleus accumbens may lead to increased depression- and anxiety like behaviour.

Finally, chronic, but not acute treatment with several classes of antidepressants (SSRIs, TADs, MAOIs) have also been shown to increase CREB phosphorylation in the cerebral cortex, hippocampus, amygdala, and hypothalamus (Thome et al., 2000).
In summary, data from post-mortem- and preclinical studies provide overwhelming evidence for a role of CREB in the pathophysiology and treatment of stress-related disorders. Some of the data, however, are mixed and more studies are warranted to define the exact role of CREB in stress and antidepressant treatment.

3.3.3.3 ERK1/2

Regulation

ERK1/2 can be activated by a variety of stimuli via RTK- and GPCR-mediated pathways (Sweatt, 2001). Furthermore, the ERKs are also sensitive to intracellular Ca\textsuperscript{2+} concentrations and may be modulated by Ca\textsuperscript{2+} dependent enzymes and/or their products, including CaMKs and NO (Schroeter et al., 2002). The phosphorylation state and activity of ERK1/2 also depend on the activity of multiple phosphatases, while its subcellular location and time-dependent activation is also important in the eventual cellular response (Murphy and Blenis, 2006). In this regard, ERK1/2 activation may be capable of promoting neuronal survival, as well as inducing neuronal death via necrosis and apoptosis (Grewal et al., 1999; Schroeter et al., 2002; Subramanian and Unsicker, 2006).

Targets

The ERKs have numerous targets including membrane, cytoplasmic and nuclear substrates. Membrane and cytoplasmic targets include cytoskeletal proteins, ion channels, receptors, phospholipase A\textsubscript{2}, components involved in their own activation and dephosphorylation (Rebay et al., 2002; Sweatt, 2004; Thomas and Huganir, 2004) and kinases such as the 90kDa ribosomal protein S6 kinases (p90RSKs) and MAPK-interacting kinases (MNKs) (Pearson et al., 2001). One activated, p90RSKs phosphorylate cytoplasmic targets such as GSK-3 and proteins involved in NF\textsuperscript{kB} and cell adhesion signalling, as well as nuclear targets such as CREB, CBP, p300, c-Fos and serum response factor (SRF) (Frödin and Gammeltoft, 1999). Activated MNKs on the other hand, remain in the cytoplasm where they participate in control of mRNA translation (Pearson et al., 2001). The ERKs themselves are also capable of translocating into the nucleus, where they can modulate gene expression directly or indirectly via the
activation of other kinases such as the mitogen stimulated kinases (MSKs), which phosphorylate transcription factors such as CREB and ATF-1, as well as proteins involved in increasing transcription factor access to DNA (Pearson et al., 2001).

**Involvement in Stress**

The ERKs play a role in synaptic plasticity, synaptic transmission and structural plasticity, as well as a contradictory role in neuronal survival (Thomas and Huganir, 2004). Thus, the ERKs are essential for neuronal survival via their actions on CREB and BAD (Bonni et al., 1999). For example, ERK activation has been shown to be involved in BDNF mediated neuroprotection against glutamate (Almeida et al., 2005), as well as in protection of neurones from death induced by neurotrophic factor withdrawal (Thiel et al., 2005). In contrast, a cell death promoting role has also been suggested for ERK1/2 via numerous effects, including suppression of Akt (Zhuang and Schnellmann, 2006). In this regard, persistent ERK1/2 activation has been shown to contribute glutamate induced neuronal injury (Stanciu et al., 2001).

Postmortem studies provide evidence for the involvement of ERK1/2 signalling in stress-related disorders, as depression is associated with a reduction in the expression and activity of ERK1/2, b-Raf and MEK, as well as an increase in MAPK phosphatase activity in the hippocampus and prefrontal cortex (Dwivedi et al., 2001; Dwivedi et al., 2006). No post-mortem studies regarding the role of ERK1/2 in PTSD have been published.

Data from pre-clinical studies are inconsistent. Similar to clinical studies, specific animal models of depression have shown significant reduction in p-ERK1/2 or p-ERK2 expression in the hippocampus and prefrontal cortex, with depressive-like behaviour correlated with these changes in MAPK signalling (Feng et al., 2003; Qi et al., 2006). No studies using specific animal models of PTSD (behavioural sensitisation) that investigated hippocampal and frontal cortex ERK1/2 have been published. Other pre-clinical stress studies using maternal separation (Huang and Lin, 2006), chronic footshock (Trentani et al., 2002), sub-chronic immobilisation (Lee et al., 2006a) and intermittent repeated exposure to social threat or a novel cage (Pardon et al., 2005) have shown hyperphosphorylation of the ERKs in brain regions involved in the stress-response,
including the amygdala, hippocampus and prefrontal cortex. In contrast, one study found chronic restraint to induce a reduction in prefrontal cortex p-ERK1/2 (Meller et al., 2003).

Regarding the molecular mechanism of antidepressants in the hippocampus and prefrontal cortex, chronic, but not acute fluoxetine treatment results in long-lasting inhibition of p-ERK1/2 expression in the nucleus but not cytosol. In the prefrontal cortex, chronic fluoxetine also inhibited p-ERK1/2 in the nucleus, with inhibition of cytosolic p-ERK2 only noted in the prefrontal cortex. Chronic imipramine treatment on the other hand, had no effect on hippocampal p-ERK1/2, but significantly upregulated prefrontal cortex p-ERK1. Neither drug had an effect on ERK1/2 phosphorylation in the striatum (Fumagalli et al., 2005). In contrast, another study found acute fluoxetine administration to significantly upregulate p-ERK in the dorsal striatum, hippocampus and prefrontal cortex (Valjent et al., 2004). In a study on the acute effects of memantine in the forced swim test, it was found that its antidepressant effect was prevented by pre-treatment with an ERK inhibitor, suggesting that ERK signalling plays a crucial role in the drug's acute antidepressant effect (Almeida et al., 2006). Finally, MEK inhibitors have been shown to have antidepressant effects in the forced swim test (Einat et al., 2003). In conclusion, the diverse data suggest ERK signalling may contribute to antidepressant action.

In summary, data from post-mortem- and preclinical studies provide substantial evidence for a role of ERK1/2 signalling in the pathophysiology and treatment of stress-related disorders, although more research is needed.

**3.3.3.4 GSK-3α/β**

GSK-3α and GSK-3β are multifunctional kinases that are downstream targets of many signalling cascades including the cAMP-PKA, PLC-PKC and PI3K-Akt cascades. GSK-3 is involved in numerous physiological processes in the brain, including neuronal growth, differentiation and survival (Kockeritz et al., 2006). Its dysregulation may therefore impair neuroplasticity and lower the ability of neurones to respond to stressful conditions (Jope and Roh, 2006).
**Regulation**

The activity of GSK-3 is regulated by phosphorylation, particularly negative regulation by phosphorylation at serine 21 (α) and 9 (β), by kinases such as Akt, PKA, PKC, p90Rsk and p70 S6 kinase (Grimes and Jope, 2001). Conversely, dephosphorylation of GSK-3 by protein phosphatases activates the enzyme. GSK-3 can also be phosphorylated at tyrosine 216 (GSK-3β) and 279 (GSK-3α) by tyrosine kinases or autophosphorylation, thereby facilitating its activity (Doble and Woodgett, 2003; Jope, 2004). The activity and effects of GSK-3 is also be regulated by other mechanisms, including binding proteins, intracellular localisation and substrate pre-phosphorylation (Jope and Roh, 2006).

**Targets**

GSK-3 has a multitude of downstream targets that includes transcription factors (CREB), cytoskeletal proteins and molecules involved in cell division (Doble and Woodgett, 2003; Picchini et al., 2004). Mostly, GSK-3-mediated phosphorylation of these substrates result in their inhibition (Doble and Woodgett, 2003), and a decrease in cellular resilience and survival (Grimes and Jope, 2001; Kaytor and Orr, 2002; Kockeritz et al., 2006). Indeed, GSK-3 has been shown to promote intrinsic apoptosis by contributing to mitochondrial disruption, promoting p53-induced expression of BAX and blocking CREB-dependent expression of BCL-2 (Beurel and Jope, 2006).

**Involvement in Stress**

Postmortem studies have implicated GSK-3 in the pathophysiology of psychiatric disorders. A recent post-mortem study found increased GSK-3β activity in the ventral prefrontal cortex of suicide and non-suicide subjects with depression compared to suicide and non-suicide subjects without depression (Karege et al., 2007). Other postmortem studies detected no difference in prefrontal cortex GSK-3β protein expression between bipolar disorder- or depression subjects and controls (Beasley et al., 2002; Lesort et al., 1999), although differences in phosphorylation levels were not measured. No postmortem studies regarding GSK-3 have been performed in PTSD.
In a genetic animal model of depression, congenital learned helplessness rats had reduced GSK-3β mRNA expression in the hippocampus compared to outbred controls (Kohen et al., 2003). GSK-3 has not been studied in any specific PTSD animal model, however, acute, subchronic, or chronic cold restraint stress has been found to have no effect on frontal cortex GSK-3β protein levels (Kozlovsky et al., 2002), although phosphorylation levels were not measured. Of interest however, is that transgenic mice overexpressing GSK-3β display memory deficits (Hernández et al., 2002), a common symptom of depression and PTSD (Austin et al., 2001; Jelinek et al., 2006). Furthermore, although these mice have normal baseline and stress-induced plasma corticosterone and ACTH levels, they show decreased habituation in the open field, increased ASR and decreased ASR habituation (Prickaerts et al., 2006).

GSK-3 signalling has also been implicated in the molecular mechanism of antidepressants. Treatment with fluoxetine and imipramine has been shown to significantly increase levels of inactive GSK3β (p-GSK3β ser 9) in the prefrontal cortex, hippocampus and striatum, a response mediated by 5-HT1A receptors, but attenuated by 5-HT2 receptors (Li et al., 2004c). Furthermore, selective inhibitors of GSK-3 have antidepressant-like effects in the forced swim test (Gould et al., 2004; Kaidanovich-Beilin et al., 2004).

In summary, postmortem and pre-clinical studies indicate a role for GSK-3, and especially GSK-3β activity, in the pathophysiology and treatment of stress-related disorders.

### 3.3.3.5 BCL-2 Proteins

#### Regulation and Role in Apoptosis

The BCL-2 family of proteins are critical regulators of the apoptosis process and consists of pro-apoptotic and anti-apoptotic proteins. Anti-apoptotic members include BCL-2, and BCL-xL, and pro-apoptotic members include BAX, BAK and BOK, which have structures similar to those of BCL-2. The pro-apoptotic group also includes BAD, BIM and BID, which are structurally different from the other BCL-2-like proteins and known as the
BH3-only proteins (Borner, 2003; Skommer et al., 2007). Balance between these pro- and anti-apoptotic proteins is important for health and that loss of this balance may lead to disease (Cotman et al., 2000).

The pro-apoptotic mechanism of BAX and BID is believed to involve the release of mitochondrial proteins such as cyt c, possibly via formation of a mitochondrial membrane pore (Robertson and Orrenius, 2002). BCL-2 and BCL-xL on the other hand, are anti-apoptotic as a result of their direct or indirect inhibition of caspases (Benn and Woolf, 2004; Borner, 2003) and possible antioxidant effects (Soane and Fiskum, 2005). Furthermore, some pro- and anti-apoptotic members are capable of heterodimerisation, thereby controlling each others effects (Hengartner, 2000). For example, BCL-2 can bind BAX, thereby keeping it in the cytoplasm and prevent the damaging effects of BAX on mitochondria (Benn and Woolf, 2004; Skommer et al., 2007). In addition, binding of BH3-only proteins such as BAD to anti-apoptotic members may free other bound pro-apoptotic members and allow their pro-apoptotic action (Skommer et al., 2007).

The activity of BCL-2 family members is tightly controlled at transcriptional, post-transcriptional and post-translational level (Skommer et al., 2007). At the transcriptional level, BDNF, p53 and glucocorticoids have been shown to induce expression of BCL-2-like proteins (Almawi et al., 2004; Almeida et al., 2005; Schuler and Green, 2005). With regard to post-transcriptional regulation, alternative splicing of BCL-2 family protein mRNA has been documented and seems to play an important functional role in apoptosis regulation (Akgul et al., 2004). Finally, the activity of many BCL-2 family members may be regulated post-translationally by cleavage by a caspase (BID), phosphorylation (BCL-2, BAD) and interactions with p53 (Adams and Cory, 2001; Moll et al., 2005; Shacka and Roth, 2005).

**Involvement in Stress**

Postmortem studies have provided evidence for the involvement of pro- and anti-apoptotic proteins in psychiatric disorders. For example, low BCL-2 expression and a high BAX : BCL-2 ratio has been demonstrated in the temporal cortex of schizophrenic subjects compared to controls (Jarskog et al., 2000; Jarskog et al., 2004). In one of these studies, subjects with depression and bipolar disorder also show a trend towards lower
temporal cortex BCL-2 levels than controls (Jarskog et al., 2000). Once again, no postmortem studies of BCL-2-like proteins have been performed in PTSD.

In a genetic model of depression, congenital learned helplessness rats displayed a significant increase in prefrontal cortex BCL-2 mRNA levels compared to outbred controls, with no significant differences in the hippocampus (Kohen et al., 2003). Although no study using a specific PTSD animal model has investigated BCL-2 family proteins, chronic restraint has been shown to induce a significant decrease BCL-2 expression in the hippocampus, which returned to normal 3 weeks after stress exposure (Luo et al., 2004). In addition, chronic prenatal restraint stress has been found to increase BAX and decrease BCL-2 expression in the hippocampus of one month old offspring (Zhang et al., 2005), but chronic restraint stress or chronic multiple stress in the adult rat was unable to replicate this effect (Liu et al., 2006). Finally, social stress has been found to exacerbate stroke outcome by suppressing BCL-2 expression (Devries et al., 2001).

Genetic mutation studies also provide support for BCL-2 family proteins in anxiety disorders. In this regard, targeted mutation of the BCL-2 gene in mice results in enhanced anxiety-like behaviour, but with normal performance in the forced swim test (Einat et al., 2005). Conversely, mice overexpressing BCL-2 demonstrate decreased fear- and anxiety related behaviour (Rondi-Reig et al., 1997), as well as decreased apoptosis and increased neurogenesis in the adult hippocampus (Kuhn et al., 2005).

*In vitro* and *in vivo* pre-clinical studies have also implicated BCL-2 family proteins in the molecular mechanism of antidepressants. For example, moclobemide increases BCL-2 and BCL-x<sub>L</sub> expression and induces differentiation in neural stem cells (Chiou et al., 2006). In rats, chronic treatment with low dose amitriptyline or venlafaxine both increase BCL-2 immunoreactivity in hippocampal mossy fibres (Xu et al., 2002). Moreover, studies consistently show that chronic treatment with lithium is neuroprotective (Chuang et al., 2002) and that the molecular mechanism underlying this effect may involve upregulation of BCL-2 protein or mRNA expression, and decreased expression of BAX in the rat hippocampus and frontal cortex (Chen et al., 1999; Li et al., 2003a).

In summary, data from pre-clinical studies provide some evidence for a role for BCL-2 family members in the pathophysiology and treatment of stress-related disorders.
3.3.3.6 NO

Regulation and Targets

NO is synthesised by three main NOS isoforms and has numerous physiological and pathological functions (Bishop and Anderson, 2005; Duncan and Heales, 2005). Whether NO acts as a physiological messenger or toxin depends on several factors including its source (NOS isoform), concentration, and the cellular environment (cell type) (Bishop and Anderson, 2005).

Both excessive and insufficient NO may have negative effects on the brain with regard to neuronal survival and resilience. For example, NO has been suggested to have both pro- and anti-apoptotic effects. In the presence of low concentrations of NO, apoptosis may be inhibited via inhibition of caspases and induction of antioxidant enzymes and heat shock protein expression (Blaise et al., 2005). In addition, physiological NO activates ERK1/2 and PI3K-Akt pathways, leading to suppression of p53 and BAX, phosphorylation and inhibition of BAD and subsequently, inhibition of cyto c release. Activation of these pathways may also have pro-survival effects through phosphorylation and activation of CREB and the subsequent upregulation of BDNF and BCL-2 expression (Bishop and Anderson, 2005; Schroeter et al., 2002). NO may also increase the functional recovery of neurones (Cárdenas et al., 2005). Overall, low or physiological concentrations of NO seem to have a beneficial effect on neuronal resilience and insufficient NO may therefore be harmful. On the other hand, high concentrations of NO may be pro-apoptotic, possibly by forming reactive nitrogenous species (RNS) and reactive oxygen species (ROS) and subsequently induction of DNA damage, p53 activation, inhibition of mitochondrial respiration and release of cyto c (Kim et al., 2001; Li and Wogan, 2005). Therefore, just like insufficient NO, pathologically high concentrations of NO may have negative effects on neurones.

Involvement in Stress

Postmortem studies indicate the involvement of NO in psychiatric disorders such as schizophrenia (Yao et al., 2004). Abnormal NO signalling has also been suggested in
stress-related disorders (Esch et al., 2002), including depression (McLeod et al., 2001) and PTSD (Pall, 2001). Postmortem studies investigating NO in these disorders are limited, with one study reporting reduced expression of NOS in the hypothalamus of depressed patients (Bernstein et al., 1998). Increased plasma NO levels have, however, been found in depressed patients that attempted suicide (Kim et al., 2006; Lee et al., 2006b).

Specific animal models for depression and PTSD have also implicated NO in the pathophysiology of these disorders. For example, chronic unpredictable stress or chronic mild stress increases serum NO levels (Fu et al., 2005) and liver NOS activity, the latter of which is reversible by chronic fluoxetine treatment (Li et al., 2003b). Chronic mild stress also induces dendritic atrophy of hippocampal neurones, which can be inhibited by fluoxetine-induced reduction of NOS expression (Luo and Tan, 2001). Learned helplessness behaviour induced by inescapable tailshock may be also be mediated by NO, as it can be blocked by dorsal raphe nucleus NOS inhibition (Grahn et al., 2000). A single prolonged stress (SPS) plus re-stress (RS) paradigm (time-dependent sensitisation model of PTSD) of behavioural sensitisation induces an increase in hippocampal nitrite and nitrate (NO metabolites) levels 7 days after the RS (Harvey et al., 2005), as well as an increase in hippocampal NOS activity 21 days after the RS (Harvey et al., 2004b). Chronic restraint stress followed by 7 days recovery also induces significant elevations in hippocampal and frontal cortex nitrite levels, although no differences in spatial learning or memory are detected at this time point (Abidin et al., 2004). Similarly, predator stress increases hippocampal NO levels and nNOS expression immediately, 12 hours and 1 day after exposure, although more long-term effects were not measured (Wang et al., 2003). Finally, chronic restraint has been shown to induce nNOS and iNOS expression in stress-related brain regions (De Oliveira et al., 2000; Olivenza et al., 2000) and to cause depression- and anxiety-like behaviour in rats immediately after stress exposure (Sevgi et al., 2006).

Genetic studies also provide evidence for the involvement of NO in stress-related disorders, as nNOS knock-out mice show reduced depressive-like and anxiety-like behaviour (Bilbo et al., 2003; Salchner et al., 2004), indicating that deletion of nNOS may alter stress-coping ability. Indeed, nNOS deletion results in higher baseline-,
suppressed stress-induced corticosterone levels, supporting a role for NO in the neuroendocrine response to stress (Bilbo et al., 2003).

Regarding antidepressant action, chronic antidepressant treatment has been reported to significantly decrease serum NO levels in patients with major depression (Herken et al., 2007) and panic disorder (Herken et al., 2006). In contrast, other studies demonstrate increased plasma NO metabolite levels as a result of chronic antidepressant treatment in patients with major depression and healthy controls (Chrapko et al., 2006; Lara et al., 2003). In pre-clinical studies, NO has been found to be involved in the antidepressant effect of memantine (Almeida et al., 2006), while various antidepressant treatments decrease hippocampal NOS activity (Wegener et al., 2003), although negative (Wegener et al., 2004) and contradictory (Suzuki et al., 2003) findings have also been reported. The data above is supported by findings that selective nNOS inhibitors have antidepressant effects alone (Heiberg et al., 2002; Joca and Guimarães, 2006), to increase the action of SSRIs (Harkin et al., 2004) and to demonstrate anti-anxiety like effects (Volke et al., 2003).

In summary, data from clinical and preclinical studies provide convincing evidence for a role of NO in the pathophysiology and treatment of stress-related disorders.
4.1 Introduction

The most relevant information regarding the psychology, biology and therapy of a psychiatric disorder is derived from the study of humans. However, studies in humans are not always possible given ethical, moral, practical or technical reasons (Cohen and Zohar, 2004). For example, in terms of the biology of a human psychiatric disorder, the behavioural dysfunction and the underlying processes in the brain may be difficult to assess in humans. The aetiology, extent, and location of the damage is unclear and the variables associated with behavioural dysfunctions cannot be controlled sufficiently, even when using patients in a clinical setting (Van der Staay, 2006). Experiments in animals however, can be conducted under standardised housing and test conditions, thereby controlling for interfering variables and enabling experimentation in ways and with sample sizes that are impossible in humans (Cohen and Zohar, 2004).

An animal model can be defined as a living experimental system used to study brain-behaviour relationships under control conditions (Van der Staay, 2006). The use of animal research is motivated by the principle that pre-clinical models will provide valuable knowledge about the nature of the human disorder that they model, ultimately leading to the improvement of certain aspects of the clinical disorder via better treatment (Wright, 2002). The acceptability or justification of animal research is based on the expectation that the findings will be meaningful, with minimal suffering of the animals (Bird and Parlee, 2000). To be meaningful, however, an animal model should be well validated especially in terms of predictive and construct validity. Furthermore, although face validity is often considered to be unimportant, it does provide a starting point for the testing of a model. Animal models of behavioural disorders, for example, face validity of behaviour may provide opportunities for predictive validity testing. Importantly, animal
models should also be reliable or replicable, and generalisable across different environments (laboratory and housing conditions), as well as across strains and species (Van der Staay, 2006).

Despite considerable research, the underlying pathophysiology of post-traumatic stress disorder (PTSD) remains largely unknown (Siegmund and Wotjak, 2007). In this regard, well-validated animal models can be essential tools in establishing the causality of stress-induced changes, investigating the underlying physiological and neuronal mechanisms, and testing potential drug treatments for their efficacy and safety (Stam, 2007a).

### 4.2 Criteria for Evaluating Relevance of Animal Models

Validation criteria are general standards that are relevant to the evaluation of any model. In general, the validity of a model refers to the extent to which the model is useful for a given purpose and in this regard, several criteria have been suggested for animal models, including different types of validity, reliability and generalisability (figure 4.1) (Geyer and Markou, 1995; Van der Staay, 2006).

In considering criteria relevant to an animal model, it is important to keep in mind that the model consists of both an independent variable and a dependent variable. The independent variable is the experimental manipulation used to induce an abnormality and can be evaluated according to face-, predictive-, construct-, etiological-, and ecological validity criteria (Geyer and Markou, 1995). The dependent variable is the measure(s) used to measure the effects of the experimental manipulation and can be evaluated according to face-, predictive-, construct-, convergent-, and discriminant validity criteria. Finally, reliability of the model is relevant to both the independent and dependent variable (Geyer and Markou, 1995).
Figure 4.1 Criteria for evaluating an animal model of behavioural dysfunction (Van der Staay, 2006).

These criteria are based on the assumption of generalisability, i.e. that extrapolations can be made from animals to humans because of the similar physiological and behavioural characteristics of species (Geyer and Markou, 1995).
4.2.1 Validity

4.2.1.1 Face Validity

Face validity refers to the similarity between the dependent variable (behaviour) exhibited by the animal and the specific symptoms of the human condition. Simply put, face validity implies that an animal model at least resembles the human disorder it is modelling (Geyer and Markou, 1995). Face validity is, however, considered to be the weakest validation criteria of all. Behaviour is often species-specific and despite it being guided by similar underlying processes, there may be little resemblance between the behaviour of animals and humans (Van der Staay, 2006). In addition, it is impossible to model symptoms of human disorders that present as a highly personal experience or have a verbal context in animals (Wright, 2002). While face validity often provides a starting point for the development of an animal model, it cannot be used to establish the validity of the model (Geyer and Markou, 1995). In turn, poor face validity, or a lack of face validity does not automatically invalidate a model (figure 4.1) (Van der Staay, 2006).

4.2.1.2 Construct Validity

Construct validity refers to the accuracy of the theoretical basis, i.e. the accuracy with which a test measures what it is supposed to measure. With regard to the independent variable, this criterion requires that the animal model be based on the same physiological and neurobiological mechanisms as the human disorder (Geyer and Markou, 1995; Koolhaas et al., 2006). Although construct validity is considered one of the most important criteria, it is often difficult to establish. A critical obstacle in establishing construct validity is the limited knowledge of the underlying pathophysiology of many human disorders and in this regard, animal research is often in advance of human research (Koolhaas et al., 2006). Thus, the overall validity and usefulness of an animal model cannot be determined solely by its degree of construct validity. As new evidence is obtained from human and animal studies, the notion of what a model is supposed to mimic or a test is supposed to measure is adapted accordingly, and so construct validity changes in the process of further development and improvement of the model (Geyer and Markou, 1995).
Convergent and discriminant validity are subtypes of construct validity and primarily refer to the evaluation of the dependent measures. Convergent validity is the degree to which a test correlates with other tests that attempt to measure the same construct. Discriminant validity is the degree to which a test measures aspects of a phenomenon that are different from other aspects of the phenomenon that other tests assess (Kalueff and Tuohimaa, 2004). Convergent or discriminant validity alone is insufficient for establishing construct validity, but evidence of both demonstrates evidence for construct validity (Geyer and Markou, 1995).

4.2.1.3 Predictive Validity

Predictive validity refers to a model’s ability to accurately predict the events under investigation. An animal model with this kind of validity allows predictions about the human condition based on the performance of the model. In pharmacology, the term predictive validity is often used in a more narrow sense and refers to the ability of an animal model to screen drugs, that is, to accurately identify the efficacy of a therapeutic agent (Wright, 2002). Predictive validity is considered to be one of the most important evaluation criteria for the validity of an animal model (Geyer and Markou, 1995).

4.2.1.4 Aetiological Validity

The concept of aetiological validity is closely related to the concept of construct validity and involves the conditions of the experimental manipulation (independent variable) and the hypothesis about the origin of the human disorder (Geyer and Markou, 1995). A model is aetio logically valid if the origin of the phenomenon in the animal and human condition is similar. However, as the cause of many human disorders remains largely unknown, aetiological validity is generally limited to hypotheses regarding a possible origin (Geyer and Markou, 1995).

4.2.1.5 Ecological Validity

More recently, the criterion of ecological validity has been suggested in the evaluation of animal models. Ecological validity is the degree to which behaviour measured in a study
imitates behaviour that occurs in natural settings. Essentially, ecological validity refers to whether findings from a study can be generalised or extended to the real world. Important factors in ecological validity are the nature of the setting, stimuli, and response (Schmuckler, 2001). For example, in an animal model of a human stress-related disorder, four important variables with regard to the ecological validity of the stressor (the stimulus) are its nature, frequency, duration and intensity (Koolhaas et al., 2006). A stressor can be either processive or systemic by nature. Processive stressors, like neurogenic (physical) or psychogenic (psychological) stressors, involve higher order cortical assessment of the situation or stimulus. Systemic stressors include metabolic-, respiratory-, cardiovascular-, immune- and cytokine challenges that may have rapid adverse reactions (Anisman and Matheson, 2005). Often stressors used in animal models are unrelated to the biology of the species and the animal does not possess the suitable adaptive mechanisms for these situations. Thus, the animal cannot adapt and stress pathology will most likely develop (Koolhaas et al., 2006). That being said however, studies have shown that it is not so much the nature of the aversive stimulus that determines stress response and stress pathology, but the degree to which the stimulus can be predicted and controlled (Anisman and Matheson, 2005; Koolhaas et al., 2006). As discussed in earlier chapters, the stress response is adaptive, helping the organism to deal with stress. Yet under certain circumstances, stress results in the development of pathology. It has been suggested that the perceived ability to control a stressful experience and to predict its outcome may influence coping behaviour and subsequently, whether or not pathology occurs (Anisman and Matheson, 2005).

Apart from the nature of the stressor, its frequency, duration and intensity should also possess ecological validity. For example, chronicity of stress is considered to be an important factor in depression (Kessler, 1997), yet many animal models of depression use a series of irregular, acute, aversive stimuli (Willner et al., 2005). In humans, exposure to acute, traumatic events may have long-term consequences. Although most people develop acute stress reactions following a stressful life event, only a percentage go on to develop acute or chronic PTSD and some even present with delayed onset PTSD (APA: DSM-IV-TR, 2002). This suggests that the time after exposure to a stressor may be an important ecological factor in animal models of PTSD (Koolhaas et al., 2006). Another important aspect of PTSD is that only a proportion of persons exposed to a trauma eventually develop PTSD. Similarly, animals display individual differences in their susceptibility to
stress, a fact that is often overlooked in animal models of PTSD. Therefore, it is suggested that stress-exposed animals be separated according to their behaviour (adaptive vs. pathological) and then compared to controls to prevent dilution of results and bias (Cohen et al., 2003; Zhang et al., 2006c).

4.2.2 Replicability and Generalisability

An aspect of animal model evaluation that is infrequently addressed is the replicability or reliability of both the independent and dependent variables. In animal models of behavioural dysfunction for example, the deficits must be reliably inducible and the resulting dysfunctions must be measurable using reliable methods.

Standardisation of housing conditions may facilitate the comparability, replicability and interpretability of animal models. Reliability of an animal model may also be enhanced if the test protocol for the dependent variable possesses proven sensitivity and adequacy for the phenomenon being measured (Van der Staay, 2006). It should be emphasised however, that the degree of housing- and test condition standardisation does not influence the chance of obtaining singular results (Würbel, 2000). In other words, a valid animal model should be able to reliably induce changes in the dependent variable, even under different housing- and test conditions. It has even been suggested that results should be considered preliminary until they have been confirmed by investigators other than those involved in the original experiment (Van der Staay, 2006). It should be mentioned that although small inter- and intra-individual variability is usually desirable, there are cases where the study of the variability could lead to a better understanding of the phenomenon and variability can therefore not always be considered as error (Geyer and Markou, 1995).

Another important aspect in evaluating an animal model is its generalisability. Extrapolation of data across species is based on the Darwinian concept of evolution, and that despite the influence of higher cognitive functions in man, basic emotional concepts are shared by humans and other mammals (Siegmund and Wotjak, 2006; Van der Staay, 2006). It is important to keep in mind, however, that an animal model is only an estimation of a human disorder and will never fully mimic it with respect to aetiology and complex symptomology (Koolhaas et al., 2006). Indeed, an implied purpose of an animal
model is that it should provide a simplification of complex events (Van der Staay, 2006). In addition to cross-species generalisation, a model should also be generalisable across different environments (housing conditions, laboratories) and tests believed to measure the same underlying traits or states (Salomé et al., 2002; Wahlsten et al., 2003). If a model meets the criterion of generalisability, it likely possesses construct validity. If, on the other hand, a model is not generalisable, it suffers from a lack of relevance (figure 4.1) (Van der Staay, 2006).

An ideal animal would therefore not only display the same phenomenon as the human condition (face validity), but would also do so through the same mechanisms (construct validity), allow predictions of the phenomena under investigation (predictive validity) and be reliable and generalisable. The initial development of animal models is often based on face validity and thereafter on construct validity, whereas its predictive validity is ultimately a sign of its practical value (Yamamoto and Une, 2002). Fulfilment of other types of validity (aetiological, ecological, discriminant, convergent) could also enhance a model's validity (Wright, 2002). The more types of validity an animal model satisfies, the greater its value and relevance to the human condition. Fulfilment of all validity criteria by an animal model is very rare, but fortunately, this is not required for successful research (Wright, 2002). Evaluating an animal model requires information regarding the purpose of the model and by limiting its purpose one can increase the confidence in its validity (Geyer and Markou, 1995). In this regard, an alternative approach to modelling a complex human disorder in its entirety involves the modelling of selected endophenotypes. Mimicking discreet symptoms or core processes of a human condition increases the probability for the two most important validity criteria, namely construct and predictive validity (Bakshi and Kalin, 2002; Siegmund and Wotjak, 2006).

The use of animal models with restricted validity, replicability and generalisibility may slow down the scientific process, and lead to the unnecessary use of research animals (Van der Staay, 2006). The publication bias of journals in favour of positive, hypothesis confirming results (Phillips, 2004) may be the reason for the slow progress in the development of new animal models. Because negative results remain unpublished and do not reach the scientific community, poor models continue to be investigated. Publication of negative findings from well-conceived and -performed studies may help researchers to
evaluate and discard invalid models and reduce the unnecessary use of animals (Van der Staay, 2006).

4.3 Animal Models of PTSD

Animal studies have used severe physical and psychological stressors and situational reminders in an attempt to model the chronic behavioural, autonomic and neuroendocrine responses seen in PTSD. Some examples include tailshock (Garrick et al., 2001), single footshock (Siegmund and Wotjak, 2006), footshock with reminders (Louvart et al., 2006), congenital learned helplessness (King et al., 2001), predator exposure (Cohen and Zohar, 2004), underwater trauma (Cohen et al., 2004), social confrontation (Stam et al., 2000), neonatal isolation plus a single prolonged stress (Imanaka et al., 2006) and stress-re-stress time-dependent sensitisation (Harvey et al., 2003; Liberzon et al., 1997). However, when considering the criteria set for PTSD models (see following section) not all of these stressors can be considered as valid.

4.3.1 Criteria for Stressors in Animal Models of PTSD

In an attempt to establish a systematic approach for evaluating the relevance of individual stress paradigms to PTSD, Yehuda and Antelman (1993) interpreted the human diagnostic criteria for PTSD (APA: DSM-IV-TR, 2002) to suit animal research. The five criteria identified are as follows:

- Even brief stressors should be capable of inducing biological and behavioural sequelae of PTSD.
- The stressor should be able to produce the PTSD-like sequelae in a dose-dependent manner.
- The stressor should produce biological alterations that persist over time, or become more pronounced over time.
- The stressor should induce biobehavioural alterations with the potential for bidirectional expression.
Interindividual variability in response to the stressor should be present as a function of prior experience, genetics or a combination of the two.

Stam (2007b) recently identified three broadly defined groups of stress paradigms that meet the majority of the abovementioned criteria, namely relatively brief sessions of electric shocks (preshock), aversive confrontations with other animals (social stress), and a combination of various individual stressors applied in one session (single prolonged stress). All of these stress paradigms induce time-dependent sensitisation or long-lasting sensitisation and mimic many of the behavioural, autonomic and hormonal changes seen in PTSD (Stam et al., 2000).

4.3.2 Stress Sensitisation Models of PTSD

Preshock paradigms typically consist of a single session or a limited number of daily sessions in which one long, or a small number of shorter foot- or tail shocks are delivered in a relatively short period (Garrick et al., 2001; Koba et al., 2001; Servatius et al., 1995; Shimizu et al., 2004). In addition, some paradigms include situational reminders of the aversive experience to enhance the sensitisation (Li et al., 2006b; Louvant et al., 2006; Pynoos et al., 1996). In social stress paradigms, animals are exposed to a predator, predator scent or an aggressive animal of the same species (Adamec and Shallow, 1993; Cohen et al., 2006a; Cohen et al., 2006b). In another form of social stress, animals are forced to watch another being shocked (Van den Berg et al., 1998). These aversive social confrontations can be presented in a single or repeated session. In the single prolonged stress (SPS) paradigm, animals are exposed successively to 2hrs of restraint, 15-20 minutes forced swimming and ether or halothane vapours until loss of consciousness (Imanaka et al., 2006; Khan and Liberzon, 2004; Liberzon et al., 1999; Takahashi et al., 2006). Some studies using this model include a re-stress (RS) consisting of forced swimming or restraint as a reminder of the initial prolonged session (Harvey et al., 2003; Harvey et al., 2006; Liberzon et al., 1997).

In all three categories of sensitisation models, differences in behavioural and other parameters (neuroendocrine, autonomic, immune etc) are measured after a period of time
(1 week up to several months) to determine the effects of stress sensitisation. In addition, the effects of several therapeutic drugs have also been tested in these models.

4.3.2.1 Behavioural Effects

Preshock, social stress and SPS models all induce sensitisation of conditioned fear responses, as well as unconditioned, generalised behavioural sensitisation to novel stressful stimuli (Stam, 2007b). The direction of the altered behavioural response depends on the context in which it is tested (Stam, 2007b). When the threat is unavoidable or inescapable, such as in the elevated plus maze (EPM) or open field, animals express reduced exploratory behaviour and enhanced immobility (Naciti, 2002; Imanaka et al., 2006; Li et al., 2006b; Louvat et al., 2005; Louvat et al., 2006; Meerlo et al., 1996; Stam et al., 2002; Takahashi et al., 2006). In contrast, if escape is an option, such as in a swim test or in an active avoidance apparatus (shuttle box), preshocked or social stressed animals often display enhanced escape behaviour (Sawamura et al., 2004; Van Dijken et al., 1992a).

Preshock, social stress and SPS models also induce increased startle response (Cohen et al., 2004; Cohen et al., 2006a; Garrick et al., 2001; Khan and Liberzon, 2004; Pynoos et al., 1996; Servatius et al., 1995), although normal or decreased startle responses have also been found (Pijlman et al., 2003). The latter discrepant findings may be because animals had not been previously habituated to the startle chamber, possibly leading to increased immobility to the novel environment and interference with the startle response (Stam, 2007b).

With regard to learning and memory, animals perform normally in tasks such as the Morris water maze (MWM) or hole-board learning paradigm days or weeks after exposure to social stress (Buwalda et al., 2005; Wang et al., 2006). In contrast, SPS with RS as a reminder induced deficits in spatial memory acquisition and consolidation as measured in the MWM (Harvey et al., 2003). However, since the initial SPS session and RS both included forced swimming, it has been suggested that the MWM task represents a model of in-context re-traumatisation (Cohen et al., 2004) and that the observed cognitive impairment may have resulted from conditioned fear (Stam, 2007b).
In stress sensitisation models, the degree of the altered behavioural response seems to depend on the intensity of the trauma (Servatius et al., 1995; Siegmund and Wotjak, 2007). In addition, some (Jeeva, 2004; Servatius et al., 1995; Van Dijken et al., 1992c) but not all studies (Adamec et al., 1998c; Cohen et al., 2004) indicate a delay in the appearance of behavioural stress sensitisation. Furthermore, once established, behavioural stress sensitisation has been shown to persist for weeks or months (Cohen et al., 2006b; Louvart et al., 2005; Louvart et al., 2006) or even increase over time (Pynoos et al., 1996; Siegmund and Wotjak, 2007). Finally, chronic treatment with clinically effective drugs such as fluoxetine, paroxetine, valproate, diazepam, chlordiazepoxide, clomipramine and imipramine have been shown to reduce behavioural sensitisation in all three categories of behavioural sensitisation (Bruijnzeel et al., 2001b; Harvey et al., 2004a; Koolhaas et al., 1990; Li et al., 2006b; Sawamura et al., 2004; Shimizu et al., 2004; Siegmund and Wotjak, 2007; Van Dijken et al., 1992b; Von Frijtag et al., 2002).

4.3.1.2 Individual Differences

Although most people develop an acute stress reaction following a traumatic event, only 10-40 % will ultimately go on to develop PTSD (APA: DSM-IV-TR, 2002; Breslau et al., 1998; De Kloet et al., 2005). Many existing animal models ignore this clinical finding and assume that all animals exposed to a stressor will inevitably develop pathology. However, similar to humans, animals display differences in their individual susceptibility to stress in terms of pathology and behaviour (Stam, 2007b).

It may be difficult to study differential vulnerability to stress sensitisation in laboratory animals even when they are outbred, because standard housing cages for animals are often small and bare (Sherwin, 2004). This unnatural environment may impair or limit behaviour and brain development, changing the ability to adapt and predisposing animals towards hyperresponsivity (Wiedenmayer, 2004; Würgel, 2001). Even with these limitations, studies using preshock- and social stress have revealed clear evidence for individual differences in long-term stress sensitisation. In a sensitisation model of footshock within-strain variance in sensitised fear (as measured by freezing) was found, with some animals remaining at low fear levels and others (43 %) developing high levels of fear (Siegmund and Wotjak, 2007). Another model of footshock reported that 50 % of
shocked animals without situational reminders, and 70% of shocked animals with situational reminders showed abnormal behavioural sensitisation as measured by the magnitude of the acoustic startle response (Pynoos et al., 1996). Similarly, 29% of tailshock exposed rats developed behavioural sensitisation as measured by nonhabituation of the acoustic startle response (Garrick et al., 2001).

Similar results have been obtained in a predator exposure model of stress sensitisation. Interestingly, this particular study measured the prevalence of extreme behaviour (well-adaptive and mal-adaptive) in the EPM for both control and stress groups, 7 days after predator exposure (Cohen et al., 2003). Mal-adaptive behaviour was defined as 0 open arm entries plus 5 minutes spent in the closed arms, whereas well-adaptive behaviour was defined as 8 or more open arm entries and less than 60 seconds spent in the closed arms. It was found that while 25.3% of stress-exposed rats displayed mal-adaptive behaviour, only 1.33% of control rats displayed mal-adaptive behaviour. Furthermore, 80% and 24.7% of the control and stress-exposed groups respectively, could be classified as well-adapted (Cohen et al., 2003). In a follow-up study by the same group, the prevalence of extreme behavioural response was examined using cut-off criteria from both EPM and acoustic startle response (ASR) data, 1, 3, 5, 7, 14 and 30 days after predator exposure. Well-adaptive behaviour was defined as more than 8 open arm entries plus less than 60 seconds spent in the closed arms, in addition to a mean startle amplitude at 110 dB of less than 1000 units plus normal habituation. Mal-adaptive behaviour was defined as 0 open arm entries plus 5 minutes spent in the closed arms, in addition to a mean startle amplitude at 110 dB of more than 1200 units plus no habituation. In the predator stress experiment, 90% of stress-exposed rats and 5% of control rats displayed mal-adaptive behaviour in the acute phase, i.e. 1 day after stress-exposure. On the same day, 0% of stress-exposed rats and 20% of control rats were well-adapted. The percentage of mal- and well-adapted rats in the control group remained relatively constant (5% vs. 20%) throughout the study. However, the prevalence of mal-adaptive behaviour in the stress-exposed group decreased from 90% on day 1 to 25% on day 7, remaining constant until day 30. Finally, the prevalence of well-adaptive behaviour in the stress-exposed group rose from 0% on day 1 to 20% on day 7 and 30% on day 30 (Cohen et al., 2004).

Together, these studies confirm the individual susceptibility of animals to stress-induced behavioural sensitisation, thereby contributing to the construct validity of the models. In
In this regard, it has been suggested that presenting data from stress-exposed groups as a mean does not represent a population similar to those with PTSD and may weaken results, leading to bias (Cohen et al., 2003; Zhang et al., 2006c).

The reasons for the abovementioned differences in individual stress susceptibility have also been investigated. As mentioned earlier, it is not just the nature of the stressor that determines if a stress response and pathology develops, but also how the animal perceives (controllability, predictability) and copes with the threat (Anisman and Matheson, 2005; Koolhaas et al., 2006). In this regard, sensitisation studies have shown that the fleeing behaviour of a rat in response to an aggressive predator and the defensive behaviour of a rat in response to attack by an aggressive same-specie animal modulate the behavioural and physiological sensitisation. A tendency to actively escape a cat predicted a smaller increase in anxiety-like behaviour (Adamec et al., 1998c) and rats showing more counter attacks in response to an aggressor had smaller reductions in the amplitude of circadian fluctuations in locomotor activity, body temperature and heart rate (Meerlo et al., 1999).

Individual characteristics prior to the stressor may also influence the stress response and stress-induced pathology. Rats can be pre-characterised by their locomotor reactivity in a novel environment (the open field) as either high reactive, or low reactive (Stead et al., 2006). These differences in open field activity appear to be stable individual qualities that are at least partially genetically determined (Mållo et al., 2007; Stead et al., 2006; Vendruscolo et al., 2006). In studies using a single session of footshock, rats pre-characterised as low reactive showed greater behavioural stress sensitisation than high reactive rats, as measured by increased immobility in a noise challenge (Geerse et al., 2006a), and low reactive rats also showed a greater blood pressure response to the noise challenge (Geerse et al., 2006a). In contrast, another study showed that chronic social stress induced a marked deficit in spatial learning in the water maze in high reactive, but not low reactive rats (Touyarot et al., 2004). Nevertheless, these results suggest that an anxiety-prone character or passive coping style may influence the risk of developing stress-related abnormalities.

Hormonal reactivity may also underlie individual differences in stress susceptibility. In a recent social stress study, extreme behavioural changes were significantly more prevalent in rats (Lewis) with a blunted hypothalamic-pituitary-adrenal (HPA) axis response to
predator stress, than in rats with normal (Sprague-Dawley) or excessively responsive (Fischer) HPA axis systems, suggesting that a blunted HPA axis response influences susceptibility to stress sensitisation. This hypothesis was supported by the finding that severe behavioural changes could be significantly reduced by exogenous administration of corticosterone before exposure to the stressor (Cohen et al., 2006). Another study, using a preshock paradigm, reported significantly higher startle sensitisation in rats with low preshock plasma corticosterone levels (Milde et al., 2003). Both studies mimic clinical observations in PTSD, where low cortisol levels in the acute aftermath of trauma, as well as low pre-trauma cortisol levels, have been suggested to be risk factors for the development of the disorder (Delahanty et al., 2000; McFarlane et al., 1997; Yehuda et al., 2000).

4.3.1.3 Endocrine Effects

Basal- and stimulated activity of the HPA axis has been investigated in all three paradigms (preshock, social stress, SPS) of stress sensitisation.

Eight days after predator exposure, Wistar rats tended to show a decline in basal plasma corticosterone, whereas Hooded rats had elevated basal plasma corticosterone levels (Adamec, 1994). In another social stress paradigm, exposure to a predator resulted in significant elevation of plasma corticosterone levels, with mal-adapted stress-exposed rats displaying significantly higher levels than well-adapted stress-exposed rats (Cohen et al., 2003). However, as mentioned earlier, the same group reported that a blunted plasma corticosterone increase in response to stress influenced susceptibility to behavioural sensitisation (Cohen et al., 2006). Social defeat resulted in a slightly elevated basal plasma corticosterone 1 week after stress exposure, but levels returned to those of controls 3 weeks after stress exposure (Stam, 2007b).

In a preshock sensitisation model, exposure to a situational reminder 41 days after the initial footshock session resulted in a significant reduction in plasma corticosterone levels in shocked rats compared to controls (Louvart et al., 2005). The finding was, however, limited to female rats (Louvart et al., 2006). In contrast, another preshock paradigm failed
to induce changes in basal plasma corticosterone levels 14 days after stress exposure (Van Dijken et al., 1993).

In summary, basal HPA activity in stress sensitisation studies seems to mimic the clinical picture of mixed findings, where normal (Baker et al., 1999; Rasmusson et al., 2001), lower (Glover and Poland, 2002; Thaller et al., 1999; Yehuda et al., 2000) and higher (De Bellis et al., 1994; Maes et al., 1998) cortisol levels have been reported.

With regard to stimulated HPA axis activity in SPS and preshock paradigms, no formal dexamethasone tests have been performed (Stam, 2007b). However, stress sensitisation of the HPA axis response has been tested after exposure to a novel or the same (homotypic) stressor. Studies consistently show sensitisation of the HPA axis in response to a novel stressor, as measured by an increase in plasma corticosterone (Johnson et al., 2002) or adrenocorticotripin (ACTH) (Van Dijken et al., 1993). Exposure to a homotypic stressor, however, has been shown to desensitise the HPA axis (Belda et al., 2004). In the SPS paradigm with a RS reminder, initial studies showed a significant decrease in corticosterone levels of rats 7 days and 21 days after the re-stress session compared to unstressed controls (Harvey et al., 2003; Naciti, 2002; Oosthuizen, 2003). A subsequent study using the same methodology, however, failed to find altered plasma corticosterone levels 7 days after the re-stress (Jeeva, 2004). Another SPS study found that the corticosterone response to a second restraint stress was also not different from controls, although the ACTH response did show a significantly greater inhibition in previously stressed rats compared to controls (Liberzon et al., 1997). The failure to find desensitisation of the HPA axis may be related to habituation, as the RS method (forced swim or restraint) was also used in the initial prolonged stress session (Stam, 2007b). In this regard, it has been shown that a similar physical context and the possibility to predict the trauma reduce the experienced stress as measured by HPA axis activity (Grissom et al., 2007; Overmier and Murison, 2005).

Studies using the SPS paradigm without the RS showed increased hippocampal glucocorticoid receptor (GR) mRNA expression 7 and 14 days after single prolonged stress exposure (Liberzon et al., 1999). Similar results were obtained in a preshock sensitisation model, where novelty-induced ACTH response and hippocampal GR binding was increased 2 weeks after stress compared to controls (Van Dijken et al., 1993).
Thus, it seems that the SPS and preshock models mimic the enhanced negative feedback sensitivity of the HPA axis activity hypotheses in PTSD (De Kloet et al., 2006; Stam, 2007b). In contrast, the social defeat paradigm indicates decreased feedback sensitivity, which is similar to major depressive disorder (Stam, 2007b). One and three weeks after social defeat, dexamethasone suppression of the plasma ACTH response to exogenous corticotrophin releasing hormone (CRH) is diminished (Buwalda et al., 1999). GR binding in the hippocampus is lower 1 week, but not 3 weeks, after social defeat (Buwalda et al., 1999), whereas it is downregulated in the parietal cortex 3 weeks after social defeat (Buwalda et al., 2001).

4.3.1.5 Autonomic Effects

Basal and stimulated autonomic responses have also been studied in stress sensitisation models (Stam, 2007b). Previous exposure to footshock has no autonomic effects 2 to 4 weeks after stress exposure. Preshock does however, induce subtle sensitisation of blood pressure and heart rate in response to novel stressors such as exposure to a noise challenge, a novel empty cage or an electrified prod in the home cage (Bruijnzeel et al., 2001a; Geerse et al., 2006b). Similarly, social defeat seems to cause autonomic hyperresponsivity to both conditioned (cage and smell of aggressor) and unconditioned (novel empty cage) stimuli, as measured by an increase in heart rate and body temperature (de Jong et al., 2005; Tornatzky and Miczek, 1994). No studies of autonomic responses in the SPS paradigm of stress sensitisation have been published.

4.3.1.6 Neuroanatomical Effects

Macroscopic morphology of brain structure has not been investigated in animal models of stress sensitisation (Stam, 2007b). However, a recent study has reported a positive relationship between hippocampal volume and trait anxiety, indicating that smaller hippocampal volumes may be associated with increased anxiety-like behaviour (Kalisch et al., 2006).

Various studies have reported an effect of stress sensitisation on the brain at cellular level. Three weeks after brief social defeat, rats display a reduction in apical dendrite length of
neurones in the CA3 region of the hippocampus, resulting in impaired long-term potentiation (Kole et al., 2004). Another study reported dendritic atrophy of hippocampal CA3 neurones immediately after restraint stress, but not 21 days later when behavioural sensitisation was still evident (Vyas et al., 2004). There is also evidence for reduced cell proliferation in the dentate gyrus 2 hours, 1 day and 3 weeks after brief social defeat (Buwalda et al., 2005; Yap et al., 2006). In contrast, a preshock paradigm found that a decrease in hippocampal cell proliferation was only evident 7 days after tailshock (Fornal et al., 2007). The functional significance of these findings is not clear, but it is possible they may contribute to increased anxiety-like behaviour in stress-sensitised rats (Bannerman et al., 2004; Stam, 2007b). In the basolateral amygdala, a single 2-hour restraint has been shown to cause a gradual (day 1 to 10) development of anxiety-like behaviour on the elevated plus-maze, accompanied by a gradual increase of dendritic spine density (Mitra et al., 2005). These findings point to the amygdala as a possible neuroanatomical basis for stress sensitisation (Stam, 2007b).

In animals, mRNA and protein products of immediate-early genes such as c-fos have been used to quantify changes in neuronal activity in post-mortem brain tissue following stress sensitisation (Hoffman and Lyo, 2002; Stam, 2007b). In the preshock paradigm, exposure to an electrified prod in the home cage 2 weeks after footshock induced a greater number of Fos-positive cells compared to controls in brain regions such as the medial prefrontal cortex, basolateral amygdala, CA1 region of the hippocampus, paraventricular nucleus of the hypothalamus and the locus coeruleus (Bruijnzeel et al., 1999). Similarly, injection with saline 7 days after social defeat significantly increased the number of Fos-positive cells compared to controls in numerous brain regions, including the medial prefrontal cortex, basolateral and central amygdala (Nikulina et al., 2004). Response of brain regions after stress-sensitisation induced by predator stress or SPS has not been investigated (Stam, 2007b).

In summary, there is some evidence in preshock, social stress and restraint stress paradigms of stress sensitisation for long-lasting alterations in amygdala and hippocampal morphology and for sensitised responsivity of cortical, amygdala, hippocampal, thalamic and brain stem areas to external stressors (Stam, 2007b).
4.3.1.7 Neurochemical Effects

Norepinephrine

Surprisingly, a limited number of studies regarding the role of norepinephrine (NE) in stress sensitisation have been published. In a preshock model, there was no significant difference in total frontal cortex NE content following a tail flick test, 10 days after stress exposure (Caggiula et al., 1989). A stressful convulsive treatment also failed to induce differences in total frontal cortex or hippocampal NE, 1 day, 1 week or 2 weeks later (Antelman et al., 1989). Similar results were obtained for total NE content in the frontal cortex 7 days after exposure to the SPS paradigm, and 7 days after introduction of an RS (Harvey et al., 2006). In the same study, however, exposure to SPS resulted in a significant increase in total hippocampal NE 7 days later, although levels returned to normal 7 days after exposure to an RS (Harvey et al., 2006). It should be mentioned however, that all of these preclinical studies measured total NE content, which is not an indication of stimulus-induced release of the neurotransmitter from nerve terminals (Stam, 2007b).

Pharmacological studies have also provided some evidence for the involvement of NE in stress sensitisation. Peripheral injection of the α2-adrenergic receptor agonist clonidine, immediately after a single immobilisation session prevented reduced immobility in the open field 1, 7 and 14 days later (Shinba et al., 2001). The results from this study suggest that decreased NE release in projection areas blocks consolidation of behavioural stress sensitisation to a novel stressor (Stam, 2007b). In a model of conditioned fear to shock-associated cues, blockade of β-adrenergic receptors with propranolol failed to affect consolidation. However, systemic administration or local basolateral amygdala injection of propranolol did disrupt reconsolidation of auditory fear conditioning measured as the percentage of freezing 2, 9 and 16 days later (Debiec and Ledoux, 2004).

In summary, studies of noradrenergic neurotransmission in stress sensitisation, although limited, support the hypothesis of enhanced activity of the noradrenergic system in PTSD (section 2.5.5.2).
Serotonin

There is limited evidence of serotonergic changes in animal models of stress sensitisation. An SPS paradigm, with or without an RS, had no effect on frontal cortex total serotonin (5-HT) or its metabolite, 5-hydroxy-indole acetic acid (5-HIAA), 7 days later. In the hippocampus, however, total 5-HT content (but not 5-HIAA) was found to be elevated 7 days after exposure to the SPS (Harvey et al., 2006; Jeeva, 2004). In contrast, total hippocampal 5-HT serotonin content (but not 5-HIAA) was reduced 7 days after the RS (Harvey et al., 2006; Jeeva, 2004). In an earlier study using the same SPS + RS paradigm, a significant increase in density and decrease in affinity of hippocampal 5-HT_1A receptors was found. In addition, while the 5-HT_2A receptor density was unaffected, prefrontal cortex 5-HT_2A receptor affinity for [^3H] ketanserin hydrochloride was significantly reduced (Harvey et al., 2003).

Pharmacological studies with 5-HT modulating drugs have also been performed in stress sensitisation models. Treatment with a 5-HT_2A receptor antagonist 10 minutes after predator stress prevented behavioural stress sensitisation, as measured in the EPM and ASR, but not the light/dark box (Adamec et al., 2004b). In a preshock model, acute and chronic treatment with the 5-HT_1A receptor agonist flesinoxan reversed footshock-induced locomotion deficits (Van Dijken et al., 1992b). Similar results were obtained in a social stress paradigm, where another the 5-HT_1A receptor agonist, viladozone, given 10 minutes after predator stress blocked behavioural sensitisation 1 week later, as measured in the acoustic startle test, but not in the elevated plus maze (Adamec et al., 2004a).

Finally, in an SPS + RS paradigm, chronic treatment with fluoxetine prevented stress sensitisation as measured in the MWM and EPM (Harvey et al., 2003; Naciti, 2002).

In summary, similar to PTSD (section 2.5.5.3), preclinical studies of stress sensitisation indicate alterations in the serotonergic system, but further research is needed to clarify the exact role of 5-HT.

Dopamine

In a preshock model, there was no significant difference in frontal cortex and nucleus accumbens total dopamine (DA) or its metabolite dihydroxyphenylacetic acid (DOPAC)
levels following a reminder shock and tail flick test, 10 days after a brief footshock session. However, the DOPAC/DA ratio in the frontal cortex was found to be significantly increased, suggesting an increase in DA turnover (Caggiula et al., 1989). In an SPS paradigm, there were no significant differences in frontal cortex DA content 7 days later, or 7 days after an RS. Frontal cortex DOPAC levels were, however, significantly increased 7 days after the SPS, but returned to control levels 7 days after an RS (Jeeva, 2004). In the hippocampus, SPS resulted in significantly increased levels of DA and DOPAC 7 days after stress-exposure, but once again, levels returned to those of controls 7 days after an RS (Jeeva, 2004; Harvey et al., 2006).

Although preliminary, these results suggest that some long-term enhancement of the dopaminergic system may be associated with stress-sensitisation, which is in accordance with the hypothesis of increased DA release and metabolism in PTSD (section 2.5.5.4).

**GABA and Glutamate**

Support for the involvement of gamma-aminobutyric acid (GABA) and glutamate in stress sensitisation is derived mainly from pharmacological studies. Behavioural sensitisation, as measured in the elevated plus maze, light-dark box and acoustic startle test, is prevented by peripheral administration of N-methyl-D-aspartate (NMDA) receptor antagonists, 30 minutes prior to, but not 30 minutes after predator stress (Adamec et al., 1998a; Blundell et al., 2005). On the other hand, injection of an NMDA receptor antagonist into the basolateral amygdala 30 minutes before predator exposure does not affect behavioural sensitisation, whereas administration 30 minutes after predator exposure partially reverses risk assessment behaviour in the elevated plus maze (Adamec et al., 1998b). Metabotropic glutamate receptor agonists may also be involved in behavioural sensitisation. In this regard, peripheral administration of group II metabotropic glutamate receptor agonists reduces the expression of behavioural sensitisation as measured in the light-dark box and a noise challenge, 1 or 2 weeks after footshock (Brujinzeel et al., 2001c; Stam et al., 2007). Thus, different aspects of behavioural sensitisation may be mediated by glutamate in different brain areas and by different receptors (Stam, 2007b). In the SPS paradigm, daily administration of the anticonvulsant topiramate, which enhances inhibitory GABA\_A receptors, for 7 days in the second week after trauma, reduced behavioural sensitisation as measured in the acoustic startle response (Khan and
Liberzon, 1999). Studies in a different laboratory using SPS + RS found significantly decreased levels of hippocampal GABA levels 21 days, but not 7 days after RS. Frontal cortex GABA levels were also unaffected 7 days after RS (Harvey et al., 2004; Myburgh, 2005).

Taken together, some stress sensitisation studies seem to suggest the involvement of glutamate and GABA, similar to the results of clinical studies in PTSD (sections 2.4.4.2 and 2.5.5.5).

In summary, animal models of stress-induced, time-dependent sensitisation mimic a number of key aspects of long-term disturbances following extreme life events in humans. They show construct validity in causing stable, long-term changes after brief but intense stressful life events. The models also show face validity, since the alterations in behavioural reactivity, hormonal, autonomic and neurochemical function show many parallels with those in PTSD. Finally, predictive validity is implied by studies demonstrating modulating effects of anxiolytics and antidepressants on behaviour (Stam et al., 2001).

4.4 Behavioural Testing of Animal Models

During development and evaluation of an animal model of behavioural dysfunction, experimental manipulation is performed based upon theory-free, or theory driven approaches. Thereafter, behavioural phenotyping is performed, where animals are tested for behavioural changes. If the hypothesised changes are confirmed, the animal model can be further characterised and validated. If, however, behavioural changes are not found, either more sophisticated tests should be employed or the development of the animal model has failed (figure 4.2) (Van der Staay, 2006).

It is important to distinguish between animal models and how we measure them. An animal test is not a model, but refers to the means by which observations regarding the dependent variable in a model are collected. For example, the EPM is not an animal model of depression or PTSD, but may measure depression- or PTSD-like symptoms in normal and experimentally manipulated animals. In some tests, the animals respond to the
test condition with a specific, unconditioned behaviour which can be modulated by experimental manipulations, such as treatment with therapeutic agents (Van der Staay, 2006).

Figure 4.2 A systematic approach to behavioural phenotyping in the development of animal models (Van der Staay, 2006).

Thus, behavioural tests measure the response of intact animals, allowing experimental manipulations to affect in vivo systems to provide an understanding of the outcome. It should be kept in mind, however, that there are a multitude of factors involved between experimental manipulations and behaviour. As a result, the effects can sometimes be unpredictable and it can be difficult to determine whether the behaviour was indeed a result of the experimental manipulation (Barth, 1997). The apparent simplicity of some test paradigms, and the ease with which data can be collected using them, is misleading (Gerlai, 2001) and these paradigms can in fact have quite complex problems. For example, there are a number of factors that contribute to successful acquisition and performance of a cognitive task. In addition to sufficient learning and memory, an appropriate response in most rodent cognitive tasks requires general motor capabilities, intact sensorimotor
processing and an appropriate response to a rewarding stimulus (Gouirand and Matuszewich, 2005). Thus, changes in these non-memory based factors can affect the outcomes of such a task without altering learning and memory processes, leading to a faulty interpretation of the data. Despite these potential drawbacks, if all variables are controlled for where possible and results are analysed critically, validated behavioural tests can offer great insight into the effects of the experimental manipulation.

Numerous behavioural tests for cognition, anxiety, arousal, conditioned fear, unconditioned fear, arousal, motivation and reward have been employed in animal models of PTSD. In the current study, the MWM, EPM and ASR tests were chosen specifically to assess the affects of an animal model of PTSD on behaviour that is known to be affected in the clinical disorder (memory, anxiety, and arousal).

### 4.4.1 The Morris Water Maze

The MWM was first developed and described some 26 years ago (Morris, 1981) and since then has become a frequently used behavioural measurement of spatial learning and memory in rats and mice. The task is based upon a reward principle, although rats can swim, they do not like water and they want to escape from it. However, this difficult task depends on the balance of motivation provided by stress vs. reward and if the stress is too high or the reward too small, the task is not attempted (Morris, 1998).

#### 4.4.1.1 Basic Protocol

Animals are placed in a large pool of water with escape from the water being achieved by finding a submerged, hidden platform. Spatial learning is assisted with extra maze cues, which should include specific cues such as black geometric shapes (salient cues) and diffuse cues such as sources of light as general orientators (non-salient cues) (Morris, 1998; Young et al., 2006).

Learning is assessed mainly by the degree to which subjects show a decrease in escape latency as a function of repeated trials. This is based on the idea that animals have developed an optimal strategy to explore their environment and escape from the water.
with minimum effort, i.e. swimming the shortest distance possible (Wenk, 2001). Further confirmation that the location of the platform has been learned is that, if the platform is removed (probe trial), rats will search the general area where the platform was located (ratio time spent in target zone or quadrant) (Schimanski and Nguyen, 2004). Numerous other parameters can also be scored as an indication of working- (ratio time spent in target zone or quadrant, target zone entry latency) and consolidated spatial memory (target zone entry latency, platform crossings).

4.4.1.2 Factors Affecting the MWM

Although the basic procedure seems relatively simple, the MWM is influenced by various factors such as differences in apparatus, training procedure and scoring parameters, as well as by the physical and behavioural characteristics of the experimental animals.

Methodological Variables

To make the task of finding the hidden platform difficult enough, the pool used should be big enough (1.8 m diameter is acceptable) and the platform not too close to the side, or the rat could conceivably find it by just circling the poolside (Morris, 1998). Water temperature can also affect water maze performance and has to be accurate to 1 degree. A temperature of 24 °C is suggested to reduce stress and prevent hypothermia, but still provide enough motivation for the escape (Morris, 1998; Stasko and Costa, 2004).

Before starting training, animals should know that escape from the pool is possible by placing the animal on the submerged platform for a short period of time at the beginning of training. In addition, each time an animal fails to escape it should be gently guided to the platform and allowed to remain there for a short period of time so that it can learn the location. Reward in the MWM should be escape from the pool onto the platform and not rescue by the experimenter. Thus, the animal has to learn that rescue is dependent on staying on the platform and to accomplish this, the experimenter must not be seen or heard by the animal when it is on the platform (Morris, 1998).
There are numerous different training and test protocols, each with different numbers of training days, trial and sessions per day, trial length, and inter-trial and -session intervals. The general paradigm consists of a 60-90 seconds training trial, with no- or a short inter-trial interval and 4 to 10 swimming trials per day. Each researcher should choose the best protocol for his or her particular experiment. For example, decreasing the number of training trials and increasing the inter-trial intervals makes the task of finding the platform more difficult and may uncover more subtle cognitive deficits (Mandel et al., 1989).

In the initial phase of training, a rodent only has to bump into the platform once to locate it and escape from the pool. To minimise or eliminate a “lucky bump” from the analysis, a sufficient number of animals per group should be used and a probe trial can be performed. A rat that knows the location of the platform will swim repeatedly over that position looking for it, indicating that it knows the location of the platform and did not find it during training by luck (Morris, 1998). It is recommended that the probe trial should not be too long (i.e. longer than the training trials), because a long probe trial may be sensitive to persistence. As persistence or behavioural inflexibility has been associated with hippocampal and prefrontal cortex dysfunction, subjects with good functioning of these brain regions may actually stop searching in the target area soon after finding no platform, but exhibit apparent decrease in spatial memory. To address this problem, one could shorten the probe trial considerably (Gerlai, 2001).

Finally, test animals should preferably also be habituated to human handling to prevent the stress of handling on the day of the test to impair MWM performance (Gerlai, 2001; Morris, 1998).

**Organismic Variables: Physical Characteristics**

Factors like body weight, physical development and age may influence swimming speed and subsequently water maze performance. Gender and species differences, as well as nutrition and infection can also influence the behaviour of animals in the MWM (D’Hooge and De Deyn, 2001). It is therefore important to use animals of the same gender and approximately the same age, weight and physical development.
As mentioned earlier, factors other than sufficient learning and memory can be required for a sufficient performance in rodent cognitive tasks. General motor capabilities, sensorimotor processing and response to a rewarding stimulus (Gouirand and Matuszewich, 2005) can all influence MWM performance. Tests can be performed to investigate whether alterations in performance were actually a result of these and other factors. For example, a cued version of the water maze (where the platform is visible) following the visible-platform task can help to determine if any differences in a rat's performance are due to motivational/emotional or sensorimotor abnormalities as opposed to memory impairment. It has been suggested however, that the design of the probe trial is flawed and biased towards falsely finding hippocampal specificity (Gerlai, 2001). Furthermore, even during visible-platform training, rodents may still make use spatial information to locate the platform (Hauben et al., 1999). This implies that impaired visible-platform MWM performance does not necessarily mean that the observed deficit was not due to non-spatial aspects of the task. On the other hand, normal visible-platform performance together with impaired hidden-platform performance does indicate that motivation/emotional or sensorimotor defects did not contribute significantly to the hidden-platform deficits (D' Hooge and De Deyn, 2001). Despite these limitations, it is standard practice to run a hidden-platform task first, followed by the cued/visible-platform task. As an additional or alternative test of locomotor ability, swim speed can be measured during training, probe and cued trials of the MWM. Animals can also be tested for general motor capability in an open field arena, whereas a sucrose solution consumption test can be used to rule out motivational deficits (Gouirand and Matuszewich, 2005).

**Organismic Variables: Behavioural Aspects**

Behaviours such as thigmotaxis (wall hugging) and floating can significantly affect performance in the MWM, although they are not necessarily dependent on hippocampal function and learning and memory (Gerlai, 2001). Thus, data from animals that display significant wall hugging or floating behaviour during the entire training period or probe trial should be eliminated from the analysis.

Although the MWM is used as a measure of spatial memory, rats can also use non-spatial strategies to reach the platform. An animal can use “place strategy”, also known as
mapping or spatial strategy, where it builds a spatial map of the platform location by using extramaze (distal) cues and swims directly towards the platform from any point of the pool. On the other hand, when no extramaze cues are available a rat can use a more general learning component, known as the procedural component (stimulus-response strategies). Non-mapping strategies based on procedural learning include praxis strategy, a learned sequence of movements which brings it to the platform, and taxis strategy, where it approaches the platform using proximal cues (D’Hooge and De Deyn, 2001). Importantly, it should be pointed out that even when extramaze cues are present, rats may learn the location of the hidden platform by using both mapping and stimulus-response strategies (Morris and Frey, 1997). To prevent taxis strategy, all cues should be visible from inside the pool, but located well outside it (only extra-maze cues, no proximal cues). To minimize praxis strategy, random starting points should be used for each trial. However, even when random starting positions are applied animals may still employ both praxis and spatial strategies to locate the hidden platform (Dalm et al., 2000).

Apart from visual cues, it has been suggested that rats may also locate the hidden platform on the basis of olfactory or auditory cues. Indeed, animals are able to use odour trails left by previous rats to locate and escape onto the platform (Means et al., 1992). To prevent this occurrence, the water should be stirred regularly between trials. Finally, a low-intensity white-noise generator can be used to prevent rats from using auditory cues to help locate the platform (Baldi et al., 2003).

4.4.1.3 Neuroanatomy of Spatial Learning and Memory

It seems that spatial learning in general and MWM learning in particular depends upon the coordinated and functionally integrated action of different brain regions, including the hippocampus, striatum, basal forebrain, cerebellum and neocortex. In this regard, damage to any of these brain regions, as well as disconnection between them, may impair MWM performance (D’Hooge and De Deyn, 2001; Okada and Okaichi, 2006; Warburton et al., 2000).

MWM performance is especially sensitive to hippocampal lesions and it has been demonstrated that an intact functional hippocampus is required for the acquisition and
retrieval of spatial information and memories during performance (working memory), as well as for temporary memory consolidation, before memories are either forgotten or become permanently consolidated (D'Hooge and De Deyn, 2001; Knowlton and Fanselow, 1998; Riedel et al., 1999). It is important to note however, that hippocampal-lesioned rats are still able to locate a hidden platform that is always located at the same distance and direction of a visible landmark. This demonstrates that although their spatial navigation abilities are impaired, their ability to use a heading cue remains intact (Pearce et al., 1998).

4.4.1.4 Neurochemistry of Spatial Learning and Memory

Glutamatergic, GABAergic, dopaminergic, cholinergic, serotonergic, and several peptidergic neurotransmitter systems all seem to be involved in learning and memory in general, and spatial learning and memory in particular (McNamara and Skelton, 1993). In the MWM task specifically, although all of these neurotransmitters have some effect, their degree of involvement in spatial learning and memory seems to differ. In this regard, a recent meta-analysis of pharmacological studies indicate that glutamatergic, dopaminergic and GABAergic neurotransmitter systems are the most critical for adequate MWM performance (reviewed in D'Hooge and De Deyn, 2001; McNamara and Skelton, 1993; Myhrer, 2003). In addition, although they seem to have a lower impact, noradrenergic, serotonergic and cholinergic activity is also needed for normal MWM performance (Myhrer, 2003). It should be mentioned that earlier studies afforded a higher impact for cholinergic neurotransmission on spatial learning and memory (McNamara and Skelton, 1993). However, the crucial role of acetylcholine in learning and memory in general may have been overstated as a result of the neurotransmitters effects on non-cognitive processes (Blokland, 1995; Myhrer, 2003). Finally, peptidergic neurotransmitters such as somatostatin, and gonadal and adrenal hormones such as testosterone and glucocorticoids also appear to play a role in MWM performance (McNamara and Skelton, 1993).
4.4.2 The Elevated Plus Maze

The EPM, introduced in 1955 (Montgomery, 1955), was validated in 1985 (Pellow et al., 1985), and has since become one of the most popular behavioural tests for anxiety (Belzung and Griebel, 2001). The maze consists of an elevated, plus-shaped, runway with two opposing arms enclosed by walls, and two open, unprotected arms. A small ledge (1cm) is sometimes added to the open arms to prevent animals from falling.

The EPM is assumed to be ethologically valid as it is based on unconditioned behavioural responses of the animal and include elements of neophobia, exploration and approach/avoid conflict (Carobrez and Bertoglio, 2005; Wall and Meisser, 2001). In other words, the animal is driven to explore the maze, but the novelty and height of the maze (Lister, 1990), the animal’s fear of open spaces (Treit et al., 1993), or the contrast between the characteristics of the arms leads to an aversion of the open arms and a preference for the closed arms (Carobrez and Bertoglio, 2005).

4.4.2.1 Basic Protocol

The animal is usually placed in the centre of the EPM facing an open arm and allowed to explore the maze freely for a certain amount of time, usually 5 minutes. Traditionally, the level of aversion or anxiety-like behaviour in the EPM is assessed with spatio-temporal parameters, including ratio open arm entries, ratio open arm time and open arm latency (Rodgers and Johnson, 1995). Other spatio-temporal and ethological parameters that have been used include ratio time spent in the maze centre, and frequency and/or duration of behaviours such as head dipping, stretched attend postures, closed arm returns, flat back approach, sniffing, grooming, rearing and defecation (Rodgers and Johnson, 1995). Risk assessment behaviours such as head dipping and stretch attend postures are generally considered to be more sensitive indications of anxiety (Rodgers et al., 1997), but use of these ethological parameters into the EPM may also make interpretation of the underlying behaviour more difficult (Wall and Meisser, 2000). It should be kept in mind that behaviour must be understood as a whole and interpreted in context (Calatayud et al., 2004). Finally, the EPM also allows control for locomotor activity, typically assessed by the number of closed arm entries (Weiss et al., 1998). It should be cautioned, however,
that factor analysis studies indicate that many of these parameters not only load on one factor (anxiety or locomotion), but frequently on two (anxiety and locomotion / anxiety and decision making) (Calatayud et al., 2004; Rodgers and Johnson, 1995; Wall and Meisser, 2001).

### 4.4.2.2 Factors Affecting EPM

EPM behaviour may be influenced by numerous variables including methodological and organismic variables, which have been found to be the main sources of inter-laboratory variation in the use of the maze (Carobrez and Bertoglio, 2005; Hogg, 1996).

**Methodological**

Construction of the EPM has been proven to influence aversive behaviour. A number of different materials have been used, including wood and perspex. Perspex may reduce the grip of the animals, and the addition of ledges around open arms has been used to prevent animals from falling. Although the material itself may not change the sensitivity of the EPM, the addition of ledges has been suggested to affect the type of anxiety expressed by the test animal (Fernandes et al., 1996; Hogg, 1996). Other differences in EPM construction such as open arm width and closed arm colour (wall) may also influence anxiety-like behaviour (Anseloni et al., 1995; Lamberty and Gower, 1996; Pereira et al., 2005). To facilitate comparison of results, it is recommended that the maze should be constructed according to the standard parameters set by Pellow and colleagues (1985) and Lister (1987) for rats and mice, respectively.

Variability in test conditions may also influence behavioural response in the EPM. For example, the illumination level (Garcia et al., 2005) or gradient of luminosity between the open and closed arms predicts the behaviour of rats (Pereira et al., 2005). This finding can be used to manipulate baseline anxiety in an attempt to enhance the sensitivity of detection of anxiogenic or anxiolytic experimental manipulations. Other procedural variables in the EPM include prior exposure to the maze, prior handling, prior stress, housing conditions and circadian rhythm or light cycle (Carobrez and Bertoglio, 2005; Lapin, 1995; Schmitt and Hiemke, 1998; Van Driel and Talling, 2005). In this regard, it
has been suggested that repeated handling of animals for several days before experimentation serves to habituate them to stress to which they are commonly subjected to in EPM testing (Hogg, 1996) and familiarity with the experimenter increases consistency of results (Van Driel and Talling, 2005).

Variability in parameters scored may also influence results of behavioural response in the EPM. The type of parameters scored (conventional vs. traditional), their definition (arm entry), and the method of scoring (manual vs. automated) may also contribute to variability (Carobrez and Bertoglio, 2005; Hogg, 1996). For example, the definition of what constitutes an arm entry can have a major impact on results. An arm entry was originally defined as the entry of an animal into an arm with all 4 paws (Lister, 1987; Pellow et al., 1985), although others have scored an arm entry when an animal enters an arm with the 2 front paws (Qi et al., 2006). Automated techniques using light beams usually count beam breaks to track an animal’s movement in the maze, and thus data may not be based on the original paw placement criteria (Hogg, 1996). Finally, scoring of ethological parameters of risk assessment, as well as temporal (minute-to-minute) analysis of behaviour may refine the sensitivity of the EPM (Carobrez and Bertoglio).

Organismic

Organismic variables such as species, strain, age, gender, and oestrus cycle/lactation have all been shown to affect the behavioural response to EPM testing (Carobrez and Bertoglio, 2005). It is therefore important to use animals of the same gender and approximately the same age in a given experiment (Doremus et al., 2004; Rodgers and Cole, 1993) and to keep such variables in mind when comparing results between laboratories or experiments.

In conclusion, recognizing and understanding variables that affect behavioural responses in the EPM can help in the design of more effective experiments and enable comparison of data between laboratories.
4.4.2.3 Neuroanatomy of Anxiety

Several brain regions have been implicated in anxiety in general (Millan, 2003) and key roles for the amygdala (Moreira et al., 2007), hippocampus (Bertoglio et al., 2006; Kjelstrup et al., 2002) and periaqueductal gray (Graeff et al., 1993) have been suggested in modulating anxiety-like behaviour in the EPM. Similar to spatial learning and memory, however, it has become clear that is not only distinct brain areas, but also neural circuits that underlie anxiety (Sandford et al., 2000).

4.4.2.4 Neurochemistry of Anxiety

A substantial number of neurotransmitters, peptides, hormones, and other neuromodulators have been implicated in anxiety in general (Steimer, 2002). In Pharmacological challenge studies in the EPM and other animal tests anxiety have strongly implicated NE (Johnston and File, 1989; Soderpalm and Engel, 1988), 5-HT (Dhomchadha et al., 2003; File et al., 1989; Martin et al., 2002; Zhang et al., 2006a) and GABA (Cole et al., 1995; Dalvi and Rodgers, 1996) in anxiety. Some evidence for the involvement of glutamate, DA, histamine, acetylcholine, cannabinoids, CRH, corticosteroids, vasopressin, oxytocin, cholecystokinin (CCK), substance P, neuropeptide Y (NPY), opioids, nitric oxide (NO), neurosteroids and neurotrophins in anxiety also exists (Korte, 2001; Landgraf, 2005; Millan, 2003; Sanford et al., 2000; Steimer, 2002).

4.4.3 Acoustic Startle Response

The acoustic startle response is characterised by rapid contraction of facial and skeletal muscles following an unexpected and intense acoustic stimulus. The ASR is an unlearned response that has been observed in many mammalian species and is thought to be protective (Koch, 1999; Koch and Schnitzler, 1997; Yeomans and Frankland, 1996). The ASR has a non-zero baseline in humans and animals, which means that the response magnitude can be increased or decreased by experimental manipulation (Koch, 1999). Indeed, the ASR is used as a non-invasive tool to study fear, anxiety, affective disturbances, sensitization and motivational states in animals and humans (Grillon and Baas, 2003).
4.4.3.1 Basic Protocol

In humans, the startle response is measured by recording the eyeblink reflex, i.e. the rapid contraction of the orbicularis oculi muscle (Grillon and Baas, 2003). In rodents, the contraction of whole-body (skeletal) muscles determines the amplitude of the startle reflex (Weiss and Feldon, 2001).

Animals are typically exposed to a series of acoustic stimuli of a certain intensity, which for rats is generally 80 dB or more above their auditory threshold (Koch, 1999). The magnitude of the response, as well as habituation can be measured. Habituation refers to the reduction in magnitude of the ASR after repeated presentation of the stimulus, either within one session (short-term) or between multiple sessions (long-term) (Koch, 1999). The animal learns that the repeated stimulus does not signify danger or anything important and subsequently ceases to waste the effort to respond (Koch and Schnitzler, 1997). Thus, habituation is a form of non-associative learning and is considered normal. Another feature of the ASR that is often measured is pre-pulse inhibition. Pre-pulse inhibition is the reduction in startle response if a non-startling stimulus is presented 30-500 ms before the startle-inducing stimulus (Koch and Schnitzler, 1997). This phenomenon does not require learning, is a normal form of plasticity of the startle reflex and is used as an operational measure for sensorimotor gating mechanisms (Fendt et al., 2001; Weiss and Feldon, 2001).

4.4.3.2 Factors Affecting the ASR

The ASR is subject to a variety of influences, methodological and organismic by nature. However, these variables usually only enhances or attenuates the ASR (Koch and Schnitzler, 1997).

Methodological

A sigmoidal function has been described for the relationship between startling stimulus intensity and response magnitude (Hince and Martin-Iverson, 2005), where an increase in the startle stimulus intensity will result in an increase the magnitude of the ASR until a
maximum response is reached. In this regard, it has been suggested that if the purpose of an experiment is to detect changes (increases or decreases) in the magnitude of the ASR, the stimulus intensity should be selected so that the responses are not at their maximum and where a ceiling effect is attained (Marable and Maurissen, 2004). High illumination levels may also increase the magnitude of the startle response in rodents (Walker and Davis, 1997), possibly due to the fact that rodents are nocturnal animals that are vulnerable in brightly illuminated environments (Grillon and Baas, 2003). Background noise has also been shown to enhance the ASR (Davis, 1974) and most protocols include a white noise background of approximately 60-65 dB to enhance the behavioural response and prevent laboratory noise from affecting behaviour. Another methodological variable is inter-trial interval, where a shorter the inter-trial interval has been shown to produce greater within-session habituation (Davis, 1970). Finally, variables in methodology such as the length of the pre-pulse, the pre-pulse startle inter-trial intervals and the intensity of the pre-pulse may also affect the measurement of pre-pulse inhibition (Fendt et al., 2001; Jones and Shannon, 2000a).

Organismic

Numerous organismic variables have been identified in the ASR including strain (Conti and Printz, 2003; Hince et al., 2005), gender (Lehmann et al., 1999), weight (Lehmann et al., 1999), diurnal rhythm (Davis and Sollberger, 1971), housing conditions (Varty et al., 2000), developmental stress (Finamore and Port, 2000; Maslova et al., 2002) and ongoing locomotion (Plappert et al., 1993; Wecker and Ison, 1986). These variables should be taken into account when performing experiments or analysing and comparing data. For example, as bodyweight will effect the magnitude of deflections of whole body movement, rats with similar weights should be used or alternatively, the ASR of each subject can be adjusted for weight (i.e. expressed per g) (Lehmann et al., 1999; Servatius et al., 2005). Ongoing spontaneous motor activity such as grooming significantly decreases ASR magnitude and in this regard, it has been suggested that data from rodents showing substantial movements at the time of stimulus presentation should be discarded (Plappert et al., 1993; Wecker and Ison, 1986).
4.4.3.3 Neuroanatomy of ASR

The primary neuronal pathway for acoustic startle is assumed to be relatively simple, with only a few synaptic relays intervening between the cochlea and motoneurons and very fast axons mediating the startle over the long conduction distance (Yeomans and Frankland, 1996). Several different models of the ASR have been proposed, involving three, four or five central synapses. All include an initial relay in the cochlear nuclear complex, an intermediate brain stem relay in the reticular formation, a long reticulospinal pathway via the medial longitudinal fasciculus and outputs via spinal cord and brain stem motoneurones (Yeomans and Frankland, 1996).

The neuronal mechanism underlying short-term habituation is not yet clear, but the connection between neurones of the cochlear nuclei and the pontine reticular nucleus has been suggested as the site at which it takes place (Koch, 1999). An alternative hypothesis of extrinsic modulation has also been suggested, where inhibitory projections from brain sites outside the primary ASR pathway attenuates sensorimotor information transfer (Koch, 1999).

Pharmacological and lesion studies in rats have implicated numerous brain regions in pre-pulse inhibition, such as the auditory cortex, basolateral amygdala, medial prefrontal cortex, hippocampus and nucleus accumbens to name but a few (Swerdlow et al., 2001).

4.4.3.4 Neurochemistry of ASR

Pharmacological and lesion studies have implicated numerous neurotransmitters in the modulation of the startle response, habituation and/or pre-pulse inhibition, including 5-HT (Kehne et al., 1992; Nanry and Tilson, 1989; Sipes and Geyer, 1994), DA (Bell et al., 2003; Davis and Aghajanian, 1976) and NE (Alsene et al., 2006; Davis et al., 1977; Fendt et al., 1994; Leaton and Casella, 1984; Walker and Davis, 2002b). Other neurotransmitters or peptides such as acetylcholine (Jones and Shannon, 2000b; Overstreet, 1977; Sipos et al., 2001), opioids (Chang et al., 1993; Davis, 1979), glutamate (Ebert and Koch, 1992; Krase et al., 1993; Miserendino and Davis, 1993) and GABA (Birnbaum et al., 1997) may also be involved (reviewed in Davis, 1980).
5.1 Animals

The study protocol was approved (04D06) and done in accordance with the guidelines stipulated by the Ethics Committee for use of experimental animals at the North-West University. All animals were maintained according to a code of ethics in research, training, diagnosis and testing of drugs in South Africa.

Male Sprague-Dawley and Wistar rats (courtesy of the Animal Research Centre of the North-West University), weighing approximately 200-220 g, 180-200 g or 150-180 g at the start of each study were randomly assigned to groups consisting of 6-12 rats per group (table 5.1).

Table 5.1 Animals used in the study.

<table>
<thead>
<tr>
<th>Study</th>
<th>Strain</th>
<th>Weight</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Behavioural validations</td>
<td>Sprague-Dawley</td>
<td>200-220 g</td>
<td>6-12</td>
</tr>
<tr>
<td>Western blot set-up (fluoxetine)</td>
<td>Wistar</td>
<td>160-180 g</td>
<td>12</td>
</tr>
<tr>
<td>TDS: behaviour + corticosterone</td>
<td>Sprague-Dawley &amp; Wistar</td>
<td>180-200 g</td>
<td>6-12</td>
</tr>
<tr>
<td>TDS: corticosterone (test naive)</td>
<td>Sprague-Dawley &amp; Wistar</td>
<td>180-200 g</td>
<td>6-12</td>
</tr>
<tr>
<td>TDS: corticosterone (test naive + saline / fluoxetine)</td>
<td>Wistar</td>
<td>160-180 g</td>
<td>12</td>
</tr>
<tr>
<td>TDS: protein expression</td>
<td>Wistar</td>
<td>180-200 g</td>
<td>12</td>
</tr>
</tbody>
</table>
The starting weight in each study was selected in such a manner that rats weighed approximately 200-220 g at the end of each study (day of behavioural testing and/or sacrifice). Thus, in the behavioural and corticosterone studies, rats of 180-200 g were selected so that after the 2 weeks of the time-dependent sensitisation (TDS) protocol, animals weighed 200-220 g. Similarly, rats weighing 160-180 g were selected in the corticosterone (i.p. saline or fluoxetine) and protein expression (i.p. saline) studies, so that at the end of the three week injection and TDS protocol, animals weighed 200-220 g (see section 5.3).

Rats were bred (outbred) and housed at the North-West University Animal Testing Centre. Rats were kept on a natural 12 hour light/dark cycle with free access to food and water and housed 6 per cage (grid-floor; dimensions: 28 cm (W), 44.5 cm (L), 12.5 cm (H)).

5.2 Study Layout

5.2.1 Behavioural Validation Studies

Male Sprague-Dawley rats weighing approximately 200-220 g were injected with the appropriate drug or vehicle and subjected to the Morris water maze (MWM), elevated plus maze (EPM) or acoustic startle response (ASR) after the indicated times (figure 5.1 A, B, C). The ASR was also validated parametrically with untreated male Sprague-Dawley and Wistar rats weighing approximately 200-220 g.
A. 0.5 mg/kg Scopolamine / Saline → MWM (training session) ↑ 30 minutes

B. 1 mg/kg β-Carboline / 0.1 M HCl in saline → EPM ↑ 30 minutes

1 mg/kg m-CPP / Saline → EPM ↑ 15 minutes

C. 8 mg/kg d-amphetamine → ASR ↑ 10 minutes

Figure 5.1 Layout of the behavioural validation studies, including details regarding drug doses, vehicles used and time administered before A. the Morris water maze (MWM); B. the elevated plus maze (EPM); C. the acoustic startle response (ASR).

5.2.2 Western Blot Set-Up Studies

Hippocampal and frontal cortex samples from control animals were used to standardise/optimise the experimental conditions for each antibody to be used, in terms of the amount of protein loaded, the concentrations and incubation times for primary and secondary antibodies, the blocking- and washing conditions, as well as the developing conditions. The method of densitometric analysis was validated by constructing a protein concentration curve. Different amounts of protein (5-35 µg) from a single rat hippocampal sample were loaded onto a gel and the density of neuronal nitric oxide synthase (nNOS) band obtained after immunoblotting detected with the ChemiDoc XRS system and Quantity One® 1-D analysis software (Bio-Rad). Finally, the ability of the standardised Western blotting conditions and validated method of densitometric analysis to detect externally induced changes in the protein expression, phosphorylation or relative activation (phospho : total ratio) was investigated. Male Wistar rats, weighing approximately 160-180 g were subjected to daily fluoxetine (10 mg/kg) intraperitoneal
injections for 21 consecutive days. All rats were sacrificed after the last injection on day 21 and their hippocampi and frontal cortices (including the dorsal-, lateral-, medial- and prefrontal cortices) rapidly dissected out, snap frozen in liquid nitrogen and stored at -86 \(^\circ\)C until use.

5.2.3 TDS Stress Studies

5.2.3.1 TDS Stress Behavioural Studies

Untreated, male Sprague-Dawley and Wistar rats were left undisturbed (controls) or subjected to the TDS model as described in section 5.3.1 and tested in either the MWM, EPM or ASR on day 7 post-RS (figure 5.2).

\[
\begin{array}{ccc}
\text{SPS} & \rightarrow & \text{RS} & \rightarrow & \text{Behavioural test} \\
\downarrow & & \downarrow & & \downarrow \\
\text{Day 0} & \rightarrow & \text{Day 7} & \rightarrow & \text{Day 14 / 7 post-RS}
\end{array}
\]

Figure 5.2 Layout of the TDS stress behavioural studies. SPS = Single prolonged stress; RS = re-stress (see section 5.3.1 for details).

5.2.3.2 TDS Stress Endocrine Studies

The hypothalamic-pituitary-adrenal (HPA) axis activity of behavioural test exposed (MWM/EPM/ASR) and test naive Sprague-Dawley and Wistar control and TDS rats was investigated by measuring plasma corticosterone levels obtained from trunk blood at sacrifice on day 7 post-RS (figure 5.3).
Finally, plasma corticosterone levels were determined on day 7 post-RS in behavioral test naive Wistar control and TDS rats that were exposed to chronic (21 days) intraperitoneal saline or fluoxetine (10 mg/kg) injections (figure 5.4).

\[
\begin{align*}
\text{Start saline / fluoxetine injections} & \rightarrow \text{SPS} \rightarrow \text{RS} \rightarrow \text{Sacrifice} \\
\downarrow & \downarrow \downarrow \\
\text{Day 0} & \text{Day 7} \text{ Day 14} \text{ Day 21 / 7 post-RS}
\end{align*}
\]

Figure 5.4 Layout of the TDS stress corticosterone studies in behavioral test naive, saline or fluoxetine injected Wistar rats. SPS = Single prolonged stress; RS = re-stress (see section 5.3.1 for details).

\section*{5.2.4 TDS Stress Protein Expression Studies}

Male Wistar rats, weighing approximately 180-200 g at the start of the study, were left undisturbed (controls) or subjected to the TDS model as described in section 5.3.1 On day 7 post-RS, animals were sacrificed and their hippocampi and frontal cortices (including the dorsal-, lateral-, medial- and prefrontal cortices) rapidly dissected out, snap frozen in liquid nitrogen and stored at -86°C until use (figure 5.5).
Figure 5.5 Layout of the TDS stress protein expression studies in behavioural test naive Wistar rats. SPS = Single prolonged stress; RS = re-stress (see section 5.3.1 for details).

5.3 Behavioural Models and Tests

This section provides details regarding, equipment, materials, protocols, parameters and statistical data analysis of the behavioural models and tests used in the study.

5.3.1 The Time-Dependent Sensitization (TDS) Model

In the current study, a TDS model of stress was implemented to induce PTSD-like sequelae in Sprague-Dawley and Wistar rats. The method follows a version of that originally described by Liberzon and colleagues (1997), but modified for our laboratory conditions and validated by Naciti (2003) and consisted of a single prolonged stress (SPS) plus a re-stress (RS).

5.3.1.1 Equipment

The following equipment was used during the TDS procedure:

- Perspex® restrainers
- Cylindrical Perspex® swim tanks
- Tempedair drier
- 5 Litre sealable plastic container
5.3.1.2 Materials and Validation

4% Halothane (SafeLine pharmaceuticals) was used in the TDS procedure. The TDS protocol has previously been validated in our laboratory (Naciti, 2002).

5.3.1.3 Protocol

Animals were exposed to a SPS that escalate in severity on the first day of testing. All the TDS experiments took place between 08h00 and 12h00 in the morning. Rats were placed in Perspex® restrainers for 2 hours with the tail-gate adjusted to keep the rat well contained without impairing circulation to the limbs. Immediately thereafter, the rats were individually placed in approximately 30 cm of ambient water (25 °C) in a cylindrical Perspex® swim tank and allowed to swim for 15 minutes. The depth of the water was adjusted to ensure that the animal was unable to use its tail as support. Following the forced swim, rats were allowed to recover in a Tempedair drier for 10 to 15 minutes before being exposed to 0.8 ml of 4% halothane vapours in a 5 litre sealed plastic container until loss of consciousness. The animal was then placed in its home cage and allowed to recover over a period of 7 days. On the seventh day after exposure to the triple stressors, the animal was re-exposed to a re-stress procedure of forced swimming for 20 minutes (RS), before being allowed to recover for 10 to 15 minutes in a Tempedair drier. Rats were left undisturbed for another 7 days and on day 7 post RS, animals were studied in either the ASR chamber, the MWM or the EPM, or sacrificed for corticosterone and neurochemical assays.

5.3.1.4 Parameters

No parameters were measured during the TDS procedure.

5.3.2 The Morris Water Maze

Spatial memory was studied using a modified version (Goldbart et al., 2003) of the MWM procedure originally described by Morris (1981).
5.3.2.1 Equipment

The MWM consisted of a dark olive-green coloured pool, 1.8 m in diameter and 0.6 m in height and constructed from a sturdy plastic material. The pool was housed in a temperature-controlled room that maintained the water temperature at ± 24°C. The pool was filled with approximately 30 cm of water and six starting points, labelled 1-2-3-4-5-6 were marked on the sidewall. All starting points were located in three quarters of the pool. A cylindrical dark olive-green coloured platform, 28 cm in height and 8 cm in diameter, is placed in the pool quadrant without a starting point, the centre of the platform being located approximately 40 cm from the sidewall. Depending on whether a visible (cued trial) or hidden (acquisition training) platform method is employed, the platform was either submerged approximately 1 cm below the surface of the water or raised the same height above the water. For the probe trial(s), the platform was removed from the pool. The MWM was surrounded by pre-set extra-maze cues, including a ladder, a lamp with a 40 W open blue light bulb pointed toward the pool, a brightly lit bench lamp pointed towards a wall and large black geometric shapes on white walls.

A digital video camera was positioned directly above the pool and the video signal relayed to a computer in an adjacent room where the experimenter monitored the rat during the trial. Accutrac Software® enabled full collection of swim activity in the maze. Zone Mapping Software® was used in the training phase, probe trial and cued trial to create a zone map necessary to measure numerous parameters. For the training phase of the task and the probe trial, the pool was divided into 4 quadrants, namely the target, left, opposite and right quadrant. In addition, a round target zone area of approximately 3 times the size of the platform was created inside the target quadrant, and another round zone inside the target zone indicated the position of the platform (figure 5.6). The position of the pool, zone map and that of the platform itself was not changed during the acquisition training or probe trial.
In the zone map for the training phase, the platform zone served as the endzone, i.e. if a rat spent more than 2 seconds on the platform, the trial ended automatically. The zone map for the probe trial contained no endzone, therefore even if the rat spent time in the platform zone (platform is removed during probe trial), the trial continued for the full 45 seconds. For the cued trial of the task, the pool was not divided into quadrants, and the zone map consisted of only the platform zone over the platform (figure 5.7). Once a rat spent more than 2 seconds in the platform zone, i.e. on the platform, the trial ended automatically.
5.3.2.2 Materials

The centrally acting anti-muscarinic, amnesic agent (−)-scopolamine hydrobromide trihydrate (Sigma-Aldrich) versus saline (Adcock-Ingram) was used in the pharmacological validation of the MWM.

5.3.2.3 Protocol

Rats were moved in their home cages to the room adjacent to the pool room and allowed to acclimatise for 1 hour. In the validation study, rats were treated with either saline or 0.5 mg/kg scopolamine (i.p.) 30 minutes prior to each training session (Hirst et al., 2006; Takahata et al., 2005). In the TDS studies, rats were left untreated (no injections). Each rat underwent 2 training sessions per day consisting of 4 trials each. Sessions were separated by 3 hours, during which rats were returned to their home cages and allowed to rest. Before the first trial, rats were placed on the platform for 20 seconds to orientate.

Data-recording and timing began with the rat being placed into the pool at one of the designated starting points (in a quadrant not containing the platform), tail first with its head facing the wall (random starting points were used for each trial). The animal was allowed to swim for 90 seconds. If it did not escape the pool, it was gently guided to the platform by the experimenter. Once on the platform, rats were allowed to remain there for an additional 15 seconds. The animal was then removed from the platform and placed into a Tempedair drier before the next trial. The inter-trial interval was approximately 120 seconds and in this time, the second rat received a training trial. In this way, a training session of four trials was completed for 2 rats, before the training session for the next two rats were started. The procedure was repeated on the second day, with the rats receiving a total of 4 sessions consisting of a total of 16 training trials in two days.

On day 3 the rats were allowed to acclimatise for 1 hour before being tested in the probe trial. Here the platform was removed from the pool and the rat allowed to swim for 60 seconds. Thereafter rats were removed from the pool, dried in a Tempedair drier and returned to their home cages. The cued trial, where the platform is raised 1 cm above the surface of the water and marked by a white ruler, was performed after a 20 minute
recuperation period. A maximum swim time of 45 seconds is now allowed, whereupon the animal was removed from the water, dried with the Tempedair drier, and returned to its cage. All training sessions in the MWM were started at 08h00. Figure 5.8 provides a summary of the complete MWM protocol.

5.3.2.4 Parameters

All parameters were measured by the Accutrac Software® system or calculated with Microsoft Excel® using measured parameters. Swim paths plotted by the Accutrac Software® system were analysed by an experimenter using the swinpaths identified by Graziano et al (2003) as a reference and served as an indication of the search strategy employed.

Acquisition Training

Table 5.2 provides a summary of the parameters determined in the training sessions of the MWM.

*Table 5.2 Parameters determined in the training phase of the MWM.*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calculation</th>
<th>Unit</th>
<th>Correlation to Acquisition</th>
<th>Correlation to Locomotion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escape latency</td>
<td>-</td>
<td>seconds</td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>Ratio time in target zone</td>
<td>time in target zone/ escape latency</td>
<td>-</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>Target zone entry latency</td>
<td>-</td>
<td>seconds</td>
<td>Inverse</td>
<td>-</td>
</tr>
<tr>
<td>Moving distance</td>
<td>-</td>
<td>cm</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Swim speed</td>
<td>Moving distance / escape latency</td>
<td>cm/s</td>
<td>-</td>
<td>Direct</td>
</tr>
</tbody>
</table>
Day 1:

1 hr acclimatization
↓
20 s on platform
↓
4 Trials (Trial = 90 s search, 15 s on platform, 120 s inter-trial interval)
↓
Rest 3 hrs
↓
4 Trials

Day 2:

1 hr acclimatization
↓
4 Trials
↓
Rest 3 hrs
↓
4 Trials

Day 3:

1 hr acclimatization
↓
Probe Trial (45 s)
↓
Rest 20-30 min
↓
Cued Trial (max 45 s)

Figure 5.8 MWM protocol.
Subtle differences in MWM strategy may, however, not be reflected in the parameters listed in the table above. Therefore, the analysis was extended and trials were categorised according to the predominant swim strategy as described by Graziano and colleagues (2003), who identified 7 swim paths commonly displayed by rodents in the MWM. These include thigmotaxis, circling, random searching, scanning, self orientating, approaching target and direct find (figure 5.9).

Thigmotaxis (T)

Circling (C)

Random Searching (RS)

Scanning (S)

Self Orienting (O)

Approaching target (A)

Direct finding (DF)

*Figure 5.9 Swim paths in training sessions of the MWM.*

Thigmotaxis involves wall hugging, where the rat swims in the periphery of the pool and constantly makes contacts with the wall. Infrequent exits from this peripheral trajectory
may result in accidental bumping into the platform. In the next strategy, circling, the rat moves away from the pool wall in a circular trajectory and the probability of encountering the platform is increased. A random searching strategy involves non-circular swimming, with jagged movements and sudden changes in direction. This wide and repeated searching of the pool often results in finding the platform. In scanning, the rat is more focussed on the centre of the pool and will turn immediately away from the pool wall if contact is made. In self orientating, a rat which has missed the platform on its first attempt will re-orientate by returning to the start or centre of the pool, before finding the platform on its second attempt. Alternatively, a rat will first orientate itself, and then find the platform on its first attempt. An approaching target strategy involves the adjustment of trajectory while approaching the platform. Finally, in a direct find, the rat swims directly to the platform (Graziano et al., 2003). The categories can be ordered in a continuum of complexity and efficacy in solving the task, with thigmotaxis the least and direct find the most effective strategy. To quantify the strategy choice data, frequencies (% of trials) of strategies were compared across groups.

**Probe Trials**

Table 5.3 gives a summary of the parameters determined in the probe trials of the MWM.

**Cued Trials**

During cued trials, only one parameter was recorded, namely escape latency time in seconds. The time taken by a rat to escape to a visible platform is indirectly correlated to its non-cognitive functioning. If a rat fails to escape to the platform within a reasonable amount of time, it may be assumed that factors such as general motor ability, visual ability or motivation may be impaired.
Table 5.3 Parameters determined in probe trials of the MWM.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calculation</th>
<th>Unit</th>
<th>Correlation to Acquisition</th>
<th>Correlation to Locomotion</th>
</tr>
</thead>
<tbody>
<tr>
<td>% time in target vs. other quadrants</td>
<td>(time in target quadrant / probe time)*100</td>
<td>%</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>% time in target zone</td>
<td>(time in target zone / probe time)*100</td>
<td>%</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>Target zone entry latency</td>
<td>-</td>
<td>seconds</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>Platform crossings</td>
<td>-</td>
<td>nr</td>
<td>Direct</td>
<td>-</td>
</tr>
<tr>
<td>Moving distance</td>
<td>-</td>
<td>cm</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Swim speed</td>
<td>moving distance / trial time</td>
<td>cm/s</td>
<td>-</td>
<td>Direct</td>
</tr>
</tbody>
</table>

5.3.2.5 Statistical Analysis of Data

Statistical analysis of data was carried out with STATISTICA (data analysis software system) version 7.1. Session data were analysed using a repeated measures analysis of variance (ANOVA) where the degrees of freedom were adjusted (Greenhouse-Geisser correction) to compensate for unequal variances and co-variances between the trials. In addition, Student t-tests, adjusted for unequal variances, were performed for each session. All data from the probe- and cued trials were analysed with Student T-tests adjusted for unequal variances, except for time spent in quadrants, which was analysed by a one-way ANOVA followed by the Dunnett test. All data are expressed as the mean ± SEM with statistical significance defined at the 95 % (p<0.05) level.

5.3.3 Elevated Plus Maze

Anxiety-like behaviour was studied using the EPM, originally developed by Handley and Mithiani (1984).
5.3.3.1 Equipment

The EPM consists of two open and two enclosed arms joined at right angles to one another in the form of “+”. It was constructed from sturdy black perspex and elevated approximately 0.6 m above the floor. A black perspex ledge of 0.5 cm was added to the sides of the open arms to prevent the rat from falling.

The maze was located in a separate room from the area where the researcher sat during the experiment, with a wooden partition and door. Lighting conditions in the room was an open 40 W blue light bulb positioned above the maze and a brightly lit desk lamp pointed away from the maze toward one wall. A digital video camera was positioned directly above the maze and the video signal relayed to a computer where Accutrac Software® enabled full collection of activity in the maze. Zone Mapping Software® was used to create a “map” of the EPM, consisting of a centre square, two open arms and two closed arms (figure 5.10).

![Zone map of the EPM as seen from above.](image)

5.3.3.2 Materials and Validation

The EPM was validated pharmacologically, using the anxiogenic agents methyl-4-ethyl-6,7-dimethoxy-beta-carboline-3-carboxylate (β-carboline DMCM) (Sigma-Aldrich) and meta-chlorophenylpiperidine (m-CPP) (Sigma-Aldrich). Saline (Adcock-Ingram) and hydrochloric acid (Merck) were used as vehicles. Methanol (Sigma-Aldrich) was used to wipe the EPM.
5.3.3.3 Protocol

Animals were moved in their home cages to the room adjacent to the experimental room and allowed to acclimatise for 1 hour. In the validation study, rats were injected intraperitoneally with 1 mg/kg β-carboline or saline (Pähkla et al., 2000), and 1 mg/kg m-CPP or vehicle (Wallis and Lal, 1998) 30 minutes and 15 minutes, respectively, before the EPM was started. In the TDS studies, rats were left untreated (no injections).

The rat was placed in the centre square of the EPM facing an open arm and allowed to explore the maze for 5 minutes under continuous surveillance with a digital video camera. During the experiment, the researcher was separated from the maze by a wooden partition. After each individual test, the animal was returned to its home cage and the maze wiped thoroughly with methanol to eliminate any odour.

5.3.3.4 Parameters

All parameters were measured by the Accutrac Software® system or calculated with Microsoft Excel® using measured parameters. An arm entry was defined as the entry of all 4 paws into an arm. The “zone” of the centre square was drawn slightly larger than its actual size, as the software tracks the rat’s centre of gravity. By doing this, it was ensured (and confirmed manually from taped footage) that an arm entry as tracked by Accutrac Software® was registered only when all 4 paws were inside an arm.

The standard spatio-temporal parameters reflecting anxiety and locomotor activity were measured. Table 5.4 provides a summary of these parameters, as well as their relation to anxiety or locomotion.

Finally, cut-off behavioural criteria based on those previously published by Cohen and colleagues (2003) were used to classify rats as well-adapted (0-60 seconds in closed arms and ≥ 8 open arm entries) or mal-adapted (0 open arm time ratio and no open arm entries).
Table 5.4 Parameters measured in the EPM

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calculation</th>
<th>Unit</th>
<th>Correlation to Anxiety</th>
<th>Correlation to Locomotor Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open arm entries</td>
<td>-</td>
<td>nr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Closed arm entries</td>
<td>-</td>
<td>nr</td>
<td>-</td>
<td>Positive</td>
</tr>
<tr>
<td>Total entries</td>
<td>open + closed entries</td>
<td>nr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ratio open arm entries</td>
<td>(open entries / total entries) *100</td>
<td>%</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>Open arm time</td>
<td>-</td>
<td>seconds</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Closed arm time</td>
<td>-</td>
<td>seconds</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total time</td>
<td>open + closed time</td>
<td>seconds</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ratio open arm time</td>
<td>(open time / total time)*100</td>
<td>seconds</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td>Open arm latency</td>
<td>-</td>
<td>seconds</td>
<td>Positive</td>
<td>-</td>
</tr>
<tr>
<td>Distance</td>
<td>-</td>
<td>cm</td>
<td>-</td>
<td>Positive</td>
</tr>
<tr>
<td>Moving time</td>
<td>-</td>
<td>seconds</td>
<td>-</td>
<td>Positive</td>
</tr>
</tbody>
</table>

5.3.3.5 Statistical Analysis of Data

All data were subjected to Student T-tests and expressed as the mean ± SEM with statistical significance defined at the 95 % (p<0.05) level using STATISTICA (data analysis software system) version 7.1., or GraphPad Prism version 4.02.

5.3.4 The Acoustic Startle Response

ASR was studied using a modified protocol of the one described by Khan and Liberzon (2004).
5.3.4.1 Equipment

ASR was measured using an automated startle system (SR-Lab Startle Response System®) from San Diego Instruments. The system consisted of a cylindrical acrylic enclosure resting on a weight-sensitive platform inside a sound- and light-attenuated chamber. The chamber was equipped with a light and ventilation fan, as well as a peephole in the front wall. A complete sound generation system inside the chamber allowed for white noise generation and adjustable background noise and was tested daily with a sound meter (Radio Shack). Body movements produced by the startle were transduced into analogue signals by a piezoelectric unit attached to the platform. Signals were collected as 200 sequential 1 ms measurements, digitised and stored on a computer by an SR-LAB programme (San Diego Instruments).

5.3.4.2 Materials and Validation

The automated ASR system was validated by using a single parametric manipulation, the intensity of the startle-eliciting stimulus (Marable et al., 2004). The results of this study were used to select the optimal test stimulus intensity for both Sprague-Dawley and Wistar rats in the final test protocol. The indirect sympatomimetic d-amphetamine (Sigma-Aldrich) versus saline (Adcock-Ingram) was used in the pharmacological validation of the ASR protocol.

5.3.4.3 Protocol

Rats remained in their home cages and were moved individually to the experimental room immediately prior to testing. In the parametric validation and TDS studies, rats were left untreated (no injections). In the pharmacological validation study, rats were injected with 8 mg/kg d-amphetamine or saline i.p. and left undisturbed for 10 minutes in their home cages before testing (Marable and Maurissen, 2004).

In the parametric validation, and also in some of the TDS stress studies, animals were placed inside the startle chamber in the cylindrical enclosure and allowed a 5 minute adaptation period at 65 dB background noise, which was continued throughout the
experiment. The test session consisted of a random selection of trials with different stimulus intensities ranging from 65 to 120 dB in 5 dB increments. Each stimulus intensity was randomly presented 10 times, so that rats were exposed to a total of 120 trials in the test session.

In the pharmacological validation and the TDS stress studies, animals were placed inside the startle chamber in the cylindrical enclosure and allowed a 5 minute adaptation period at 65 dB background noise, which was continued throughout the experiment. Test session started with 30 startle trials of 40 ms 155 dB noise bursts with an inter-trial interval of 30 seconds. Two minutes after the last startle trial, 10 pre-pulse inhibition trials were presented in which a 40 ms 85 dB noise burst (pre-pulse) preceded the onset of a 40 ms 115 dB noise burst by 100 ms. The inter-trial interval was 30 seconds. The reflexive movements of the animal were monitored for a 200 ms period following the onset of the stimulus.

5.3.4.4 Parameters

Parameters that were measured automatically by the SR-LAB Software included V start (amplitude at onset of startle) and V max (maximum startle amplitude attained measured for 200 ms from onset of startle stimulus). Trials with a V start of 20 mV or higher indicated movement of the animal at the onset of the noise burst and were discarded from data analysis. All other parameters were calculated using V max with Microsoft Excel® (table 5.5). Parametric validation was carried out on both Sprague-Dawley and Wistar strains and as bodyweight has been shown to affect startle amplitude (Lehmann et al., 1999), V max at different stimulus intensities were corrected for weigh.

To quantify habituation of V max, the 30 startle trials were divided into 6 blocks of 5 trials and the mean maximum startle determined for each block. Habituation was then calculated between the first and last blocks. To quantify pre-pulse inhibition, the average V max for the first 10 startle trials was compared to the average V max of the 10 pre-pulse trials. Both habituation and pre-pulse inhibition were expressed as a percentage (table 5.5).
behaviourally mal-adapted rats were identified using cut-off criteria for the ASR based on those previously published by Cohen et al. (2004) (nonhabituation, i.e. ≤ 0%). Well-adapted rats showed normal habituation (Sprague Dawley ≥ 50%; Wistar ≥ 35%). These values were based on data published for normal rats of both strains (Fujiwara et al., 2006; Lehman et al., 1999; Malone et al., 2006; Varty et al., 1999; Varty et al., 2000).

### 5.3.4.5 Statistical Analysis of Data

Statistical analysis of data was carried out with STATISTICA (data analysis software system) version 7.1., or GraphPad Prism version 4.02. Trial and block data of all studies were analysed using an ANOVA, and Student T-tests were performed for each trial and block. Habituation and pre-pulse inhibition data were also subjected to Student T-tests. All data are expressed as the mean ± SEM with statistical significance defined at the 95% (p<0.05) level.
5.4 Corticosterone

The effects of TDS stress, chronic saline and chronic fluoxetine injections on plasma corticosterone were studied using a commercially available $^{125}$I radioimmunoassay (Diagnostic Products Corporation).

5.4.1 Equipment

- Heparin tubes (BD Vacutainer systems)
- Bench-top centrifuge (Sigma-Aldrich)
- Gamma counter (Packard)

5.4.2 Materials

Coat-A-Count rat corticosterone kits (Diagnostic Products Corporation) were used and contained the following:

- Rat corticosterone Ab-coated tubes
- $^{125}$I rat corticosterone
- Rat corticosterone calibrators (0-2000 ng/ml)

5.4.3 Protocol

The radioimmunoassay procedure was performed according to the manufacturer's instructions. Briefly, trunk blood was collected in heparin tubes immediately after decapitation and stored briefly on ice before centrifugation (5000 g) at 4 °C for 10 minutes. Plasma (supernatant) was removed from each tube and stored at -20 °C until use. On the day of the assay, samples were removed from storage, allowed to come to room temperature and mixed by gentle inversion. 50 ul of calibrators or samples were pipetted in duplicate into separate marked tubes. Thereafter, 1 ml of $^{125}$I rat corticosterone was added to every tube, vortexed and incubated for 2 hours at room temperature. Thereafter,
tubes were decanted thoroughly and allowed to drain for 2 to 3 minutes on absorbent paper. Tubes were counted for 1 minute in a gamma counter.

### 5.4.4 Parameters

Corticosterone values were calculated using the standard curve derived from the calibrators (0-2000 ng/ml) provided in the kit, and expressed as ng/ml.

### 5.4.5 Statistical Analysis of Data

All data were analysed with Student T-tests, except for data from the saline vs. fluoxetine study, which was subjected to a one-way ANOVA and Bonferroni tests post-hoc (comparing selected groups). GraphPad Prism version 4.02 was used and all data are expressed as the mean ± SEM with statistical significance defined at the 95 % (p<0.05) level.

### 5.5 Protein Expression

The effect of TDS stress on hippocampal and frontal cortex expression of selected proteins was determined by Western blot.

#### 5.5.1 Equipment

The following equipment was used for Western blotting experiments:

- Teflon homogeniser
- Sonicator
- Benchtop centrifuge (Sigma-Aldrich)
- Plate reader (Labsystems)
- Block heater (Stuart Scientific)
- PowerPac™ basic power supply (Bio-Rad)
- Mini-PROTEAN® 3 cell (Bio-Rad)
- Trans-Blot® SD semi-dry transfer cell (Bio-Rad)
- ChemiDoc XRS system with Quantity One® 1-D analysis software (Bio-Rad)

### 5.5.2 Materials

Table 5.6 lists the reagents used in Western blotting experiments.

**Table 5.6 Western blotting reagents.**

<table>
<thead>
<tr>
<th>Reagent/Drug</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamid/Bis solution (30 %) 29:1</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Akt antibody</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Aprotonin</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>BAX antibody</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>BCL-2 antibody</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Bradford reagent</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Fluka</td>
</tr>
<tr>
<td>Cling wrap</td>
<td>Local retailer</td>
</tr>
<tr>
<td>CREB antibody</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>ECL Advance Western blotting detection kit</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>ECL Western blotting detection kit</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>EDTA</td>
<td>Merck</td>
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<tr>
<td>Sodium phosphate monobasic dihydrate</td>
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<td>Sigma-Aldrich</td>
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<td>Trizma (Tris) base</td>
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Trypsin inhibitor

<table>
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<th>Sigma-Aldrich</th>
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</thead>
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<tr>
<td>Tris-hydrochloride (Tris-Cl)</td>
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<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Tween-20 10%</td>
</tr>
<tr>
<td>Bio-Rad</td>
</tr>
</tbody>
</table>

5.5.3 Protocol

5.5.3.1 Sample Preparation

Hippocampi and frontal cortices (including the dorsal-, lateral-, medial- and prefrontal cortices) were removed from -86 °C storage and immediately placed on ice. Samples were homogenised with a Teflon homogeniser in 1 ml ice-cold sample buffer 1 (NOS1; BCL-2; BAX; CREB; phospho-CREB; ERK1; GSK-3α/β) or buffer 2 (Akt; phospho-Akt; phospho-ERK1/2; phospho- GSK-3α/β), depending on the antibody to be used.

Sample buffer 1 contained 9.1 mM sodium phosphate dibasic dihydrate, 1.7 mM sodium phosphate monobasic dihydrate, 150 mM sodium chloride (pH 7.4 with sodium hydroxide), 1 % Nonidet P-40, 0.5 % sodium-deoxycholate, 1 % sodium dodecyl sulphate, 10 µl/ml (of 10 mg/ml stock) phenylmethylsulfonyl fluoride, 1 µl/ml (of 1 mg/ml stock) aprotinin and 5 µl/ml (of activated 200 mM stock) sodium orthovanadate. Sample buffer 2 contained 50 mM HEPES, 100 mM potassium chloride, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mg/ml aprotinin, 2 mg/ml trypsin inhibitor and 5 µl/ml (of 200 mM stock) phenylmethylsulfonyl fluoride.

Brain samples homogenised with sample buffer 1 were incubated on ice for 30 minutes followed by a 10 second sonication at low power. Thereafter, samples were vortexed, 10 µl removed for Bradford protein determination, and the rest stored at -20 °C in 50 µl aliquots until use. Brain samples prepared with sample buffer 2 were centrifuged (800 g) at 4 °C for 10 minutes immediately after homogenisation and the supernatant saved. Of the supernatant, 50 µl was removed for Bradford protein determination, and the rest stored at -86 °C in 50 µl aliquots until use.
In addition to TDS and control samples, untreated hippocampi and frontal cortices were prepared in the same way to create a “standard” sample. This standard sample was loaded onto every gel and ultimately used to normalise density data and minimise between-blot variation.

5.5.3.2 Bradford Protein Determination

Aliquots of prepared samples were set aside and used for protein level determination with the Bradford assay, which is based on complexing of proteins with Brilliant Blue G. Using a 96-well plate, 5 μl of standards (0.1-1.4 mg/ml BSA in ddH2O), 5 μl of sample and 5 μl of sample buffer (1 or 2) were pipetted in triplicate in separate wells. Bradford reagent (250 μl) was added to each well, the plate immediately mixed on a shaker and allowed to incubate at room temperature. After 15 minutes, the absorbance was read at 560 nm in a plate reader and the protein concentration of the unknown samples determined.

5.5.3.3 SDS-PAGE Electrophoresis and Immunoblotting

On the day of the assay, samples were removed from storage, allowed to thaw at room temperature and mixed in a 1 : 1 ratio with loading buffer containing 62.5 mM Tris-HCl, 2 % SDS, 25 % glycerol, 0.01 % bromophenol blue and mercaptoethanol (50 μl to every 950 μl of buffer). Thereafter, samples were boiled at 94 °C for 5 minutes using a block heater, vortexed and loaded onto 1 mm thick resolving gels.

Eight samples, plus the standard sample and protein standard were loaded onto every gel. The sequence of loading was kept constant and consisted of alternating control and TDS samples, with the “standard” sample in the middle of the gel. The amount of protein loaded (as determined with the Bradford assay) and the % of the gel depended on the antibody used (table 5.7).
Table 5.7 Polyacrylamide gel % and protein concentrations loaded.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Gel %</th>
<th>Protein concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>10%</td>
<td>30 μg</td>
</tr>
<tr>
<td>p-Akt (Thr308)</td>
<td>10%</td>
<td>40 μg</td>
</tr>
<tr>
<td>BAX</td>
<td>12%</td>
<td>10 μg</td>
</tr>
<tr>
<td>BCL-2</td>
<td>12%</td>
<td>10 μg</td>
</tr>
<tr>
<td>CREB</td>
<td>10%</td>
<td>30 μg</td>
</tr>
<tr>
<td>p-CREB (Ser133)</td>
<td>10%</td>
<td>30 μg</td>
</tr>
<tr>
<td>ERK1</td>
<td>12%</td>
<td>10 μg</td>
</tr>
<tr>
<td>p-ERK1/2 (Thr202/Tyr204)</td>
<td>12%</td>
<td>30 μg</td>
</tr>
<tr>
<td>GSK-3α/β</td>
<td>12%</td>
<td>10 μg</td>
</tr>
<tr>
<td>p-GSK-3α/β (Ser21/9)</td>
<td>12%</td>
<td>30 μg</td>
</tr>
<tr>
<td>nNOS</td>
<td>8%</td>
<td>10 μg</td>
</tr>
</tbody>
</table>

Polyacrylamide resolving gels were made by mixing 30 % acrylamide/bisacrylamide, ddH2O, 1.5 M Tris-HCl and 10 % SDS. Polymerisation of the resolving gel was then initiated by adding 10 % ammonium persulphate (APS) and N’N’N’N-Tetramethyletylenediamine (TEMED). The monomer was gently stirred and applied between the glass plates filling 3/4 of the gel sandwich. The gel solution was then immediately coated with a thin layer of water-saturated butanol to assist in polymerisation and to allow even spread of the resolving gel. After leaving the gel to polymerise for approximately 40 minutes, the butanol was carefully poured off, without disturbing the resolving gel. To remove any traces of butanol that might be present, the surface of the polymerised resolving gel was rinsed with the electrophoresis tank buffer. A 3.9 % stacking gel was prepared by mixing 30 % acrylamide/bisacrylamide, ddH2O, 0.5 M Tris-HCl, 10 % SDS, 10 % APS and TEMED. The gel sandwich was then filled to the top with the stacking gel solution and a 1 mm Teflon comb inserted to allow well formation. The gel was left to polymerise at room temperature for 1 hour, whereafter the Teflon comb was removed and the wells rinsed with electrophoresis tank buffer (0.1 % SDS, 0.25 M Tris base, 1.92 M glycine, pH 8.3).
After loading, proteins were separated by SDS-PAGE electrophoresis, by running the gel for approximately 80 minutes at 100 volts in the Mini-PROTEAN® 3 cell (Bio-Rad). Electrophoresis was terminated when the tracking dye front reached the bottom of the resolving gel. The gels were removed from the electrophoresis cell and equilibrated in transfer buffer (39 mM glycine, 48 mM Tris HCl, 0.0375 % SDS and 20 % methanol) for 30 minutes. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot® SD semi-dry transfer cell (Bio-Rad) for 30 minutes at 20 volts.

Blocking, washing, incubation and ECL conditions depended on the antibody used (table 5.8). PVDF membranes were incubated in 5 % w/v non fat dried milk or 2 % advanced blocking solution in wash buffer (10 mM Tris-Cl pH 7.5, 100 mM NaCl, 0.1 % Tween-20) for 2 hours at room temperature or overnight at 4 °C to prevent any non-specific binding of the antibodies to the membrane. The membranes were then washed (3 x 10 minute washes) by gentle agitation on a rotary shaker or simply rinsed.

Primary antibodies were diluted in 5 % w/v non-fat dry milk, 5 % BSA or 2 % advanced block and incubations were carried out at room temperature for 1 hour or overnight at 4 °C. Thereafter, each membrane was washed 4 times (15 minute wash followed by 3 x 5 minute washes) or 6 times (6 x 5 minute washes) with wash buffer by gentle agitation on a rotary shaker. Membranes were then incubated with the appropriate secondary antibody (goat anti-rabbit-, rabbit anti-goat- or goat anti-mouse horseradish peroxidase (HRP)-labelled antibody) in 5 % w/v non-fat dry milk, 5 % BSA or 2 % advanced block. Finally, each membrane was washed 4 times (15 minute wash followed by 3 x 5 minute washes) or 6 times (6 x 5 minute washes) with wash buffer by gentle agitation on a rotary shaker.
Table 5.8 Blocking and incubation conditions for the different antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>ECL</th>
<th>Block</th>
<th>Primary</th>
<th>Secondary</th>
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</thead>
<tbody>
<tr>
<td>Akt</td>
<td>Normal</td>
<td>5 % milk 1 hour RT</td>
<td>5 % BSA overnight 4 °C</td>
<td>5 % milk 1 hour RT</td>
</tr>
<tr>
<td>p-Akt (Thr308)</td>
<td>Normal</td>
<td>5 % milk 1 hour RT</td>
<td>5 % BSA overnight 4 °C</td>
<td>5 % milk 1 hour RT</td>
</tr>
<tr>
<td>BAX</td>
<td>Advanced</td>
<td>2 % advanced overnight 4 °C</td>
<td>2 % advanced 1 hour RT</td>
<td>2 % advanced 1 hour RT</td>
</tr>
<tr>
<td>BCL-2</td>
<td>Advanced</td>
<td>2 % advanced overnight 4 °C</td>
<td>2 % advanced 1 hour RT</td>
<td>2 % advanced 1 hour RT</td>
</tr>
<tr>
<td>CREB</td>
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<td>5 % BSA overnight 4 °C</td>
<td>5 % milk 1 hour RT</td>
</tr>
<tr>
<td>p-CREB (Ser133)</td>
<td>Normal</td>
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<td>5 % milk overnight 4 °C</td>
<td>5 % milk 1 hour RT</td>
</tr>
<tr>
<td>ERK1</td>
<td>Normal</td>
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<td>5 % milk 1 hour RT</td>
<td>5 % milk 1 hour RT</td>
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<tr>
<td>p-ERK1/2</td>
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<td>5 % BSA 1 hour RT</td>
<td>5 % milk 1 hour RT</td>
</tr>
</tbody>
</table>

Protein standards were marked with fluorescent tracker tape, the membranes placed onto cling wrap and incubated in normal or Advanced ECL detection solution according to the manufacturer's instructions. The solution was drained and the membranes covered with cling wrap, avoiding any trapped air bubbles, and exposed to Hyperfilm™ ECL for 15 seconds to 7 minutes in a darkroom. The films were developed immediately using Premix developer and fixed using Premix fixer. Immediately following film development, PVDF membranes with still active ECL detection solution were rapidly transferred onto the transilluminator of the ChemiDoc XRS gel documentation system and an image captured using the chemiluminescence application and selecting the Live acquire mode. Exposure
was set to collect an image every 15 seconds for a period of 1 hour. As soon as all bands on the blot were clearly visible the image collection was stopped and the last image used for data analysis. Images of exposed films were also captured with the ChemiDoc XRS system using the epi white illumination application and selecting the Auto expose mode. Both chemiluminescent- and film images were analysed by Quantity One® 1-D analysis software (Bio-Rad).

### 5.5.4 Parameters

Densities of bands (intensity/mm²) were measured by Quantity One® 1-D analysis software (Bio-Rad) and normalised against the density of the standard sample loaded onto every gel. All values were expressed as % of control.

### 5.5.5 Statistical Analysis of Data

All data were subjected to Student T-tests using GraphPad Prism version 4.02, and expressed as the mean ± SEM with statistical significance defined at the 95 % (p<0.05) level.
6.1 Introduction

The aim of the study was to determine the effect of the time-dependent sensitisation (TDS) model on cognitive, aversive and acoustic startle (arousal) behaviour of Sprague-Dawley and Wistar rats, using automated behavioural analysis systems. Three well-established animal behavioural tests, namely the Morris water maze (MWM), elevated plus maze (EPM) and acoustic startle response (ASR) paradigms were initially validated for conditions in our laboratory. All three tests were validated pharmacologically in Sprague-Dawley rats, and the ASR was also validated parametrically in Sprague-Dawley and Wistar rats. After successful validation, the MWM, EPM and ASR were used to test for cognitive impairment, anxiety and abnormal acoustic startle response, respectively, in control and TDS stress exposed Sprague-Dawley rats. When TDS stress was found to have no marked effect in any of the three behavioural tests, the experiments were repeated in control and TDS stress exposed Wistar rats, a strain known to have a more pronounced anxiety-like profile and an increased response to stress compared to Sprague-Dawleys (Bekris et al., 2005; Rex et al., 2004; Staples and McGregor, 2006).

6.2 Behavioural Test Validation Studies

6.2.1 The Morris Water Maze

The aim of this study was to validate the MWM protocol for our laboratory conditions by means of a pharmacological challenge with a known amnesic drug.
6.2.1.1 Parameters

During the training phase of the task, escape latency time, ratio time spent in target zone, target zone entry latency time, swim speed and swim strategy were determined for each session. Escape latency and target zone entry latency are inversely correlated, and ratio time in target zone directly correlated to acquisition of spatial memory. Swim speed gives an approximation of general motor capability, whereas swim path analysis provides an indication of the search strategy employed. In the probe trial, percentage time spent in quadrants, percentage time spent in target zone, target zone entry latency time, platform crossings and swim speed were determined. Target quadrant bias is sometimes used as an indication of spatial memory consolidation. Ratio time spent in target zone and platform crossings are also directly correlated to spatial memory consolidation, whereas target zone entry latency is indirectly correlated with spatial memory consolidation. Once again, swim speed gives an approximation of general motor capability. Finally, escape latency time was measured in the cued trial and serves as an indication of non-cognitive functioning such as general motor capability, visual capability and motivation (Gerlai, 2001; Hodges, 1996; Schimanski and Nguyen, 2004).

6.2.1.2 Treatments

The MWM protocol was validated with the centrally acting anti-muscarinic agent, scopolamine, a drug known to inhibit spatial learning and memory (Choi et al., 2006; Diez-Ariza et al., 2003; Janas et al., 2005). Rats were allowed to acclimatise for 1 hour in their home cages in the room adjacent to the pool and injected (i.p.) with either saline or 0.5 mg/kg scopolamine, 30 minutes prior to each training session.

In should be mentioned that the use of systemic scopolamine in the validation of the MWM has been criticised, due to its possible interference with some of these non-cognitive processes (Myhrer, 2003). A crucial question is whether cognitive effects of systemic scopolamine are overshadowed by non-cognitive interference. In this regard, although systemic administration of 0.5 mg/kg scopolamine has been shown to impair non-spatial visual memory, possible non-mnemonic effects of the drug like discrimination deficit, motivational deficit and locomotor dysfunction has been ruled out (Andersen et
Despite concerns, there exists substantial evidence indicating that systemic administration of scopolamine does indeed impair MWM spatial learning and memory (Choi et al., 2006; Diez-Ariz a et al., 2003; Janas et al., 2005) and systemic scopolamine administration continues to be used widely in MWM studies in doses ranging from 0.2 to 2 mg/kg (Fadda et al., 2006; Fan et al., 2005; Hirst et al., 2006; Janas et al., 2005; Komater et al., 2005; Takahata et al., 2005).

6.2.1.3 Acquisition Training

Escape Latency

A two-way ANOVA applied to escape latency times with sessions as a repeated measure showed that there were no significant session-by-group interaction, but that there was a significant session effect over the two groups \([F(2.149; 21.486)=7.502, p=0.0029]\). Finally, T-tests adjusted for unequal variances were performed for each session and the scopolamine treated group was found to have statistically significant longer escape latencies at session 3 \([t(6.218)=-2.816 (p=0.0294)]\) and 4 \([t(8.751)=-4.887 (p=0.0009)]\) compared to the saline group (figure 6.1).

![Figure 6.1 Effects of acute scopolamine treatment on escape latency times (per session) as measured in the MWM maze (mean ± SEM; n=6; Saline ---; Scopolamine ○ ---; *p<0.05 vs. Saline).](image)
Ratio Time Spent in Target Zone

A two-way ANOVA applied to ratio time spent in target zone with sessions as a repeated measure showed that there were no significant session-by-group interaction, but there was a significant session effect over the two groups \( F(2.580; 25.802)=3.953, p=0.0234 \). Finally, T-tests adjusted for unequal variances were performed for each session and the scopolamine treated group was found to have a statistically significant lower ratio time in target zone at session 4 \( t(9.957)=3.966 (p=0.0027) \) compared to the saline group (figure 6.2).

![Figure 6.2](image)

Figure 6.2 Effects of acute scopolamine treatment on ratio times in target zone (per session) as measured in the MWM maze (mean ± SEM; n=6; Saline ■ —; Scopolamine o ---; * p<0.05 vs. Saline).

Target Zone Entry Latency

A two-way ANOVA applied to target zone entry latency times with sessions as a repeated measure showed that there was no significant session-by-group interaction, but that there was a significant trial effect over the two groups \( F(2.120; 21.204)=6.393, p=0.0060 \). Finally, T-tests adjusted for unequal variances were performed for each session and the scopolamine treated group was found to have a statistically significant higher target zone latency.
entry latency at session 1 \( t(6.436)=-3.420 \ (p=0.0127) \) compared to the saline group (figure 6.3).

Figure 6.3 Effects of acute scopolamine treatment on target zone entry latency times (per session) as measured in the MWM maze (mean ± SEM; \( n=6; \) Saline ■ ---; Scopolamine ----; \( p<0.05 \) vs. Saline).

**Swim Speed**

A two-way ANOVA applied to swim speeds with sessions as a repeated measure showed that there were no significant session-by-group interaction and no significant trial effect over the two groups. Finally, T-tests adjusted for unequal variances were performed for each session and differences in mean swim speeds between groups were not statistically significant (figure 6.4).
Figure 6.4 Effects of acute scopolamine treatment on swim speed (per session) as measured in the MWM maze (mean ± SEM, n=6; Saline ■ —; Scopolamine ○ ---).

Swim Strategy

T-tests adjusted for unequal variances were performed for the percentage of training trials where each specific search strategy was used. Differences in mean percentage of trials where thigmotaxic- [t(5.73)=-2.95 (p=0.0269)], circling-[t(8.1)=-2.81 (p=0.0225)], self orientating-[t(8.53)=3.16 (p=0.0124)] and approaching target [t(6.85)=3.21 (p=0.0152)] strategies were employed were statistically significant between groups. Scopolamine treated rats displayed thigmotaxic- and circling search strategies in a statistically higher percentage of trials compared to saline treated rats. Scopolamine rats also made use of self orientating- and approaching target strategies in a statistically lower percentage of trials than saline treated rats (figure 6.5).
Figure 6.5 Effects of scopolamine treatment on the frequency of search strategy type used over all 16 training trials of the MWM (mean ± SEM; n=6; * p<0.05 vs. Saline).

6.2.1.4 Probe Trial

Time Spent in Target vs. Other Quadrants

A one-way ANOVA revealed no statistically significant differences between the means of percentage time spent in target quadrant and percentage time spent in the left, opposite or right quadrants for the saline (figure 6.6 A) or scopolamine (figure 6.6 B) groups.
Figure 6.6 Quadrant preference of the saline (A) and scopolamine (B) groups, as measured in the probe trial of the MWM (mean ± SEM, n=6).

**Time Spent in Target Zone**

T-tests adjusted for unequal variances showed that there was a statistically significant difference in the means of percentage time spent in target zone between the two groups, with scopolamine treated rats spending significantly less time in the target zone [t(8.133)=2.366 (p=0.0451)] (figure 6.7).

Figure 6.7 Effect of acute scopolamine treatment on percentage time in target zone as measured in the probe trial of the MWM (mean ± SEM; n=6; *p<0.05 vs. Saline).
**Target Zone Entry Latency**

T-tests adjusted for unequal variances showed that there was a statistically significant difference in the means of target zone entry latency between the two groups, with scopolamine treated rats taking significantly longer to enter the target zone \( t(6.925) = -2.444 \) \((p=0.0449)\) (figure 6.8).

![Figure 6.8 Effect of acute scopolamine treatment on target zone entry latency as measured in the probe trial of the MWM (mean ± SEM; n=6; * p<0.05 vs. Saline).](image)

**Platform Crossings**

T-tests for unequal variances showed that there were no statistically significant differences in the means of platform crossings between the two groups, although scopolamine treated rats tended to cross the platform zone less (figure 6.9).

![Figure 6.9 Effect of acute scopolamine treatment on platform crossings as measured in the probe trial of the MWM (mean ± SEM; n=6).](image)
**Swim Speed**

T-tests for unequal variances showed that there were no statistically significant differences in the swim speed between the two groups (figure 6.10).

![Swim Speed Graph](image)

*Figure 6.10 Effect of acute scopolamine treatment on swim speed as measured in the probe trial of the MWM (mean ± SEM; n=6).*

**6.2.1.5 Cued Trial**

**Escape Latency**

T-tests adjusted for unequal variances showed that there were statistically significant differences in the means of escape latency time between the two groups, with scopolamine treated rats taking significantly longer to find the platform than their saline controls \[t(7.722)=-9.800 \ (p<0.0001)\] (figure 6.11).

![Escape Latency Graph](image)

*Figure 6.11 Effect of acute scopolamine treatment on escape latency time as measured in the cued trial of the MWM (mean ± SEM; n=6; * p<0.05 vs. Saline).*
6.2.2 The Elevated Plus Maze

The aim of the study was to validate the EPM for our laboratory conditions by means of a pharmacological challenge with known anxiogenic drugs.

6.2.2.1 Parameters

Parameters recorded or calculated included anxiety-related parameters (ratio open arm entries, ratio open arm time, open arm latency) and locomotor-related parameters (closed entries, distance, moving time).

6.2.2.2 Treatments

Animals were moved in their home cages to the room adjacent to the experimental room and allowed to acclimatise for 1 hour. Thereafter, rats were injected (i.p.) with 1 mg/kg β-carboline or saline (Pähkla et al., 2000), and m-CPP or vehicle (Wallis and Lal, 1998) 30 minutes and 15 minutes, respectively, before the EPM was started.

The non-selective inverse benzodiazepine receptor agonist, methyl-4-ethyl-6,7-dimethoxy-beta-carboline-3-carboxylate (β-carboline DMCM; 1 mg/kg) (Pähkla et al., 2000), was chosen. However, although the drug induced anxiety-like behaviour in the EPM, it also affected locomotor activity. Subsequently, the validation was confirmed with another anxiogenic drug which did not affect locomotor activity, namely the preferential 5-HT2C receptor agonist, meta-chlorophenylpiperidine (m-CPP; 1 mg/kg) (Wallis and Lal, 1998).

6.2.2.3 Anxiety-Related Behaviour

Open Arm Entries

T-tests revealed statistically significant differences in percentage ratio open arm entries between vehicle and drug treated groups, with the β-carboline [t(10)=3.2084 (p=0.0094)]
(figure 6.12 A) and m-CPP \([t(9)=3.3305 \ (p=0.0088)]\) (figure 6.12 B) treated rats entering less often into the open arms of the EPM than their respective controls.

**Open Arm Time**

T-tests revealed statistically significant differences in percentage ratio open arm time between the groups, with the \(\beta\)-carboline \([t(10)=3.2668 \ (p=0.0085)]\) (figure 6.13 A) and m-CPP \([t(9)=2.5125 \ (p=0.0332)]\) (figure 6.13 B) treated rats spending less time in the open arms than their respective controls.

**Figure 6.12** Effects of \(\beta\)-carboline (A) and m-CPP (B) treatment on ratio open arm entries in the EPM (mean ± SEM; \(\beta\)-carboline, Vehicle, m-CPP \(n=6\); Saline \(n=5\)).

**Figure 6.13** Effects of \(\beta\)-carboline (A) and m-CPP (B) treatment on ratio open arm time in the EPM (mean ± SEM; \(\beta\)-carboline, Vehicle, m-CPP \(n=6\); Saline \(n=5\)).
Open Arm Latency

T-tests revealed no statistically significant differences in open arm latency between vehicle and β-carboline (figure 6.14 A) or m-CPP (figure 6.14 B) treated groups, although rats treated with the anxiogenic drugs did tend to demonstrate a trend toward longer open arm latencies than their respective controls.

![Open Arm Latency Graph]

Figure 6.14 Effects of β-carboline (A) and m-CPP (B) treatment on open arm latency in the EPM (mean ± SEM; β-carboline, Vehicle, m-CPP n=6; Saline n=5).

6.2.2.4 Locomotor Activity

Closed Arm Entries

A t-test revealed a statistically significant difference in closed arm entries between the β-carboline and vehicle treated group [(t(10)=3.2668 (p=0.0085)], with the β-carboline rats making significantly more entries into the closed arm of the EPM (figure 6.15 A). There was, however, no statistically significant difference in closed arm entries between the m-CPP and sample treated groups (figure 6.14 B)
**Distance and Moving Time**

T-tests revealed no statistically significant differences in distance or moving time between the drug-treated groups and their respective controls (data not shown).

**6.2.3 The Acoustic Startle Response**

The aim of the parametric validation was to determine the optimal stimulus intensity to detect any possible difference in ASR in the TDS stress studies. The aim of the pharmacological validation was to confirm the sensitivity of the protocol used.

**6.2.3.1 Parameters**

In the parametric validation study, rats were subjected to a random selection of trials with different stimulus intensities ranging from 65 to 120 dB in 5 dB increments. Each stimulus intensity was randomly presented 10 times, so that rats were exposed to a total of 120 trials in the test session, and the mean maximum startle for each intensity calculated. In the pharmacological validation study, rats were subjected to 30 startle trials followed by 10 pre-pulse trials during which numerous parameters including maximum startle over
all 30 trials, baseline startle, total startle, mean startle over blocks, habituation of startle and pre-pulse inhibition of startle were determined.

### 6.2.3.3 Treatments

In the pharmacological validation study, rats were injected (i.p.) with 8 mg/kg d-amphetamine or saline and left undisturbed for 10 minutes in their home cages before testing (Marable and Maurissen, 2004). D-amphetamine releases biogenic amines from their storage sites in nerve terminals (Hoffman, 2001) and has been shown to potentiate ASR and disrupt pre-pulse inhibition in rats (Bell et al., 2003; Johansson, et al., 1995; Marable and Maurissen, 2004).

### 6.2.3.4 Parametric Validation

A two-way ANOVA applied to maximum startle values (corrected for bodyweight) with stimulus intensity trials as a repeated measure showed that there was a significant trial-by-group interaction [F(2.605; 57.328)=6.3082, p=0.0015], as well as a significant trial effect over the two groups [F(2.605; 57.328)=116.885, p>0.000001]. Finally, T-tests adjusted for unequal variances were performed for each stimulus intensity and Sprague-Dawley rats were found to have statistically significant higher maximum startle values at 65 dB [t(19.666)=2.130 (p=0.0459)], 90 dB [t(12.884)=3.157 (p=0.0076)], 95 dB [t(16.506)=2.585 (p=0.0196)] and 100 dB [t(12.730)=2.854 (p=0.0138)], but statistically significant lower maximum startle values at 115 dB [t(20.812)=2.680 (p=0.0141)] and 120 dB [t(21.686)=2.332 (p=0.0294)] compared to Wistar rats (figure 6.16).
6.2.3.5 Pharmacological Validation

6.2.3.5.1 Startle Amplitude

Maximum Startle Over Individual Trials

A two-way ANOVA applied to maximum startle (V max) with trials as a repeated measure showed that there was neither a significant trial-by-group interaction, nor a significant trial effect over the two groups. Finally, T-tests adjusted for unequal variances were performed for each trial and differences in mean V max between groups were statistically significant at trials 13 [t(7.676)=2.531 (p=0.0364)], 15 [t(10.323)=4.623 (p=0.009)], 20 [t(9.224)=2.73 (p=0.0485)], 21 [t(9.480)=2.467 (p=0.0345)] and 25 [t(10.922)=2.514 (p=0.0289)] (figure 6.17). Thus, d-amphetamine evoked a statistically significant increase in mean V max at many of the test trials.
Figure 6.17 Effects of d-amphetamine on maximum startle over individual trials (mean ± SEM; Saline ■ — n=6; d-amphetamine ○ --- n=7).

**Baseline Startle**

T-tests showed that there were no statistically significant differences in the means of the baseline startle (first 5 trials) between the two groups (figure 6.18).

Figure 6.18 Effect of d-amphetamine on baseline startle (mean ± SEM; Saline n=6; d-amphetamine n=7).
**Average Total Startle**

T-tests showed that there were no statistically significant differences in the means of the total startle (over all 30 trials) between the two groups, although the total startle of the amphetamine group did tend to be higher (figure 6.19).

![Graph showing total startle means](image)

*Figure 6.19 Effect of d-amphetamine on total startle (mean ± SEM; Saline n=6; d-amphetamine n=7).*

### 6.2.3.5.2 Habituation of Startle

A two-way ANOVA applied to mean maximum startle (V max) with blocks as a repeated measure showed that there was no significant block-by-group interaction, but that there was a significant block effect over the two groups \([F(1.5272;16.7987)=7.1471, p=0.0089]\). Finally, T-tests revealed no statistically significant differences in mean V max between groups at any block, although there was a tendency toward increased mean V max in d-amphetamine treated rats across all blocks (figure 6.20).
Figure 6.20 Effects of d-amphetamine treatment on mean maximum startle over 6 blocks (mean ± SEM; Saline ■ — n=6; d-amphetamine ○ --- n=7).

T-tests also revealed no statistically significant differences in the mean percentage habituation from block 1 to 6 between the two groups, although percentage habituation tended to be lower in the d-amphetamine group compared to the saline group (figure 6.21).

Figure 6.21 Effect of d-amphetamine on percentage habituation (from block 1 to 6) (mean ± SEM; Saline n=6; d-amphetamine n=7).

6.2.3.5.3 Pre-Pulse Inhibition of Startle

T-tests showed statistically significant differences in mean percentage pre-pulse inhibition (first 10 startle trials vs. 10 PPI trials) between the groups, with d-amphetamine
significantly disrupting pre-pulse-inhibition compared to saline \([t(6.97)=2.79 \ (p=0.0272)]\) (figure 6.22).

![Figure 6.22 Effect of \(d\)-amphetamine on pre-pulse inhibition of maximum startle](image)

**6.2.4 Synopsis**

The following section provides a summary and brief discussion of the results obtained in the behavioural test validation studies.

**6.2.4.1 MWM**

In the pharmacological validation study of the MWM, rats were treated with either saline, or 0.5 mg/kg scopolamine, a drug known to disrupt spatial memory, 30 minutes before each training session of the MWM (Hirst et al., 2006; Takahata et al., 2005).

Scopolamine treatment resulted in a statistically significant increase in escape latency and target zone entry latency, as well as a statistically significant decrease in ratio time spent in target zone in some training sessions, compared to saline treatment. Rats treated with scopolamine employed inferior search strategies (thigmotaxis and circling; Graziano et al., 2003) in significantly more training trials than saline treated controls. This is in accordance with previous studies showing scopolamine to typically induce thigmotaxis (Hodges, 1996). In addition, scopolamine treated rats displayed some effective search strategies (self-orientation and approach target) in significantly fewer trials than saline.
treated rats. There were no statistically significant differences in swim speed between groups in any of the training sessions, indicating a lack of drug-induced locomotor alterations. In the probe trial, percentage time in target zone was significantly less and target zone entry latency significantly longer in scopolamine rats than saline controls. Scopolamine treated rats also tended to make fewer platform zone crossings than saline controls. Again, there were no statistically significant differences in swim speed between the groups.

Together, the abovementioned results confirm that scopolamine treatment impaired spatial memory acquisition and consolidation. However, in the cued trial, scopolamine treatment resulted in significantly longer escape latency than saline treatment. As the cued trial is often used to detect non-cognitive functioning (Choi et al., 2006), these results indicate a possible effect of scopolamine on non-cognitive functions (Gouirand and Matuszewich, 2005), which, alone or together with a cognitive deficit, may be responsible for the observed impaired MWM performance of scopolamine treated rats. However, impaired cued-trial performance does not necessarily mean that the observed deficit relates solely to non-cognitive aspects of the MWM task. Indeed, studies demonstrate a failure of systemically administered scopolamine to induce non-mnemonic effects like discrimination deficit, motivational deficit and locomotor dysfunction (Andersen et al., 2002). Drug-induced locomotor dysfunction can be excluded, as there were no statistically significant differences in swim speed between groups in the training sessions or probe trial. In addition, it has been documented that even during visible-platform training rodents still appear to use spatial information to locate the platform (Hauben et al., 1999). Despite concerns about the non-cognitive effects of systemic scopolamine, substantial evidence indicates that systemic administration of scopolamine does indeed impair MWM spatial learning and memory (Choi et al., 2006; Diez-Ariza et al., 2003; Janas et al., 2005) and as such, continues to be used widely in MWM studies (Fadda et al., 2006; Hirst et al., 2006; Janas et al., 2005; Komater et al., 2005; Takahata et al., 2005; Schreiber et al., 2006). Therefore, although non-cognitive, non-locomotor deficiencies cannot be ruled out, the current data conclude that poor MWM performance of scopolamine treated rats is related to the amnesic effect of the drug. After consideration of these observations, the MWM protocol was deemed sufficiently validated and used unchanged in the subsequent TDS stress behavioural studies.
One important observation, however, was that neither the saline, nor scopolamine rats displayed target quadrant preference or spent significantly more than 25% of the total probe trial time in the target quadrant. These parameters are often used to confirm good spatial memory consolidation (Gerlai, 2001) and the use of a place-learning strategy to solve the MWM task, as opposed to taxis or praxis strategies (Baldi et al., 2003; D’Hooge and De Deyn, 2001; McNamara and Skelton, 1993). Therefore, the lack of target quadrant bias point to the possibility that saline treated rats, either failed to learn the task (memory acquisition and/or consolidation), or solved the task (finding the platform) by employing a strategy other than place learning. Although the reason for the possible failure in learning the task or employment of non-place learning strategies is unknown, it might be related to possible pre-existing anxiety or other characteristic in rats used in the study (see chapter 9).

6.2.4.2 EPM

In the pharmacological validation study of the EPM, rats were treated with one of two known anxiogenic drugs, 1 mg/kg β-carboline or 1 mg/kg m-CPP or vehicle controls, 30 or 15 minutes before the EPM, respectively (Pähkla et al., 2000; Wallis and Lal, 1998).

Locomotor activity, as measured by the number of closed arm entries, was increased in the β-carboline group compared to the vehicle control group. As alterations in locomotor activity may confound interpretation of anxiety-related parameters in the EPM, the validation of the EPM was confirmed with a second anxiogenic drug (m-CPP), which was found to have no effect on locomotion.

Treatment with either β-carboline or m-CPP resulted in an increase in anxiety-related behaviour in the EPM, as measured by a statistically significant decrease in ratio open arm time and ratio open arm entries in the EPM compared to vehicle controls. There were, however, no significant differences is open arm latency, although β-carboline and m-CPP treated rats did tend to take longer to enter an open arm compared to control rats.
These results confirm the anxiogenic effects of \( \beta \)-carboline and m-CPP and the EPM protocol was therefore considered validated and used unchanged in the subsequent TDS stress studies.

6.2.4.3 ASR

6.2.4.3.1 Parametric Validation

In the parametric validation study of the ASR, Sprague-Dawley and Wistar rats were left untreated (no injections). Because bodyweight can have a significant effect on ASR and Sprague-Dawleys are usually heavier than Wistars, maximum startle values were corrected for weight. Analysis of results shows a statistically significant trial-by group interaction as well as a significant trial effect. Sprague-Dawley rats displayed significantly higher maximum startle amplitudes at 65, 90, 95 and 100 dB, but significantly lower maximum startle amplitudes at 115 and 120 dB than those for Wistar rats. The observation of a higher maximum startle amplitude in Wistar rats compared to Sprague-Dawley rats is in accordance with the results of a recent study comparing ASR of these two strains (Hince and Martin-Iverson, 2005).

The results from the parametric study were used to help design the protocol for the pharmacological validation and TDS stress studies in both strains. The purpose of these studies was to detect any variations (increases or decreases) in maximum startle and therefore, a sub-maximal stimulus intensity was selected (115 dB) to prevent a ceiling effect (Marable and Maurissen, 2004).

6.2.4.3.1 Pharmacological Validation

In the pharmacological validation study of the ASR, rats were treated with either saline or 8 mg/kg d-amphetamine, a drug known to potentiate acoustic startle amplitude and disrupt pre-pulse inhibition, 10 minutes before the ASR (Marable and Maurissen, 2004).

There was no statistically significant trial-group interaction, but d-amphetamine treatment did result in a statistically significant up-regulation of maximum startle at trials 13, 15,
20, 21 and 25. In addition, there was no statistically significant trial effect over the two groups, suggesting a possible lack of startle habituation from trial to trial for both saline and d-amphetamine treated rats. When baseline startle and total startle were compared, there were no statistically significant differences although total startle tended to be higher in d-amphetamine treated rats than saline controls. d-Amphetamine appeared to have no significant effect on mean maximum startle over the 6 blocks. Indeed, there were no statistically significant differences in percentage habituation from block 1 to 6 between d-amphetamine and saline treated rats. Finally, the d-amphetamine treated group did show a statistically significant disruption in pre-pulse inhibition compared to the saline control group.

The abovementioned results are in accordance with previous studies that show d-amphetamine treatment to increase maximum startle and disrupt pre-pulse inhibition, whilst having no significant effect on startle habituation (Johansson et al., 1995; Marable and Maurissen, 2004; Sills et al., 1999). The ASR protocol was therefore considered validated and used unchanged in the subsequent TDS stress studies.

It should be pointed out, however, that the mean habituation of 30.804% observed for the saline group was noticeably lower than the 53-60% reported in the literature for normal Sprague-Dawley rats (Malone et al., 2006; Varty et al., 1999; Varty et al., 2000).

6.3 TDS Stress Studies: Sprague-Dawleys

The aim of this study was to determine the effects of TDS stress on cognitive ability, aversive behaviour and arousal of Sprague-Dawley rats, by using validated animal tests, namely the MWM, EPM and ASR, respectively.

6.3.1 The Morris Water Maze

TDS stress exposed and control Sprague-Dawley rats were moved in their home cages to the room adjacent to the pool room and allowed to acclimatise for 1 hour. Rats were not exposed to any injections. The parameters measured were the same as those in the pharmacological validation study.
6.3.1.1 Acquisition Training

Escape Latency

A two-way ANOVA applied to escape latency times with sessions as a repeated measure showed that there was no significant session-by-group interaction, but that there was a significant session effect over the two groups [F(2.072; 33.148)=38.100, p<0.000001]. Finally, T-tests adjusted for unequal variances performed for each session revealed no statistically significant differences in mean escape latency times between groups (figure 6.23).

![Graph showing escape latency times](image)

Figure 6.23 Effects of TDS stress in Sprague-Dawley rats on escape latency times (per session) as measured in the MWM (mean ± SEM; Control ■ n=6; TDS ○ n=12).

Ratio Time Spent in Target Zone

A two-way ANOVA applied to ratio time spent in target zone with sessions as a repeated measure showed that there was no significant session-by-group interaction, but that there was a significant session effect over the two groups [F(2.783; 44.534)=17.556, p<0.000001]. Finally, T-tests adjusted for unequal variances, performed for each session
showed that there were no statistically significant differences in mean ratio time spent in target zone between groups (figure 6.24).

![Graph showing ratio time in target zone over sessions for control and TDS groups.](image)

*Figure 6.24 Effects of TDS stress on ratio time spent in target zone (per session) as measured in the MWM (mean ± SEM; Control ■ — n=6; TDS ○ — n=12).*

**Target Zone Entry Latency**

A two-way ANOVA applied to target zone entry latency times with sessions as a repeated measure showed that there was no significant session-by-group interaction, but that there was a significant session effect over the two groups \[F(2.083; 33.334)=16.364, \ p<0.000009\]. Finally, T-tests adjusted for unequal variances, performed for each session showed that there were no statistically significant differences in mean target zone entry latency times between the two groups (figure 6.25).
Figure 6.25 Effects of TDS stress in Sprague-Dawley rats on target zone entry latency (per session) as measured in the MWM (mean ± SEM; Control ■ — n=6; TDS ○ --- n=12).

Swim Speed

A two-way ANOVA applied to swim speed with sessions as a repeated measure showed that there was neither a significant session-by-group interaction, nor a significant session effect over the two groups. Finally, T-tests adjusted for unequal variances performed for each session showed no statistically significant differences in mean swim speeds (figure 6.26).
Figure 6.26 Effects of TDS stress in Sprague-Dawley rats on swim speed (per session) as measured in the MWM (mean ± SEM; Control ■ — n=6; TDS ○ --- n=12).

**Swim Strategy**

T-tests adjusted for unequal variances were performed for the percentage of training trials where each specific search strategy was used. There were no statistically significant differences for any swim strategy between control and TDS rats (figure 6.27).
Figure 6.27 Effects of TDS stress in Sprague-Dawley rats on the frequency of search strategy type used over all 16 training trials of the MWM (mean ± SEM; Control n=6; TDS n=12).

6.3.1.2 Probe Trial

Time Spent in Target Quadrant

A one-way ANOVA revealed no statistically significant differences between the means of percentage time spent in target quadrant and percentage time spent in the left, opposite or right quadrants for the Control (figure 6.28 A) or TDS (figure 6.28 B) groups.
Figure 6.28 Quadrant preference of the Sprague-Dawley Control (A) and TDS (B) groups, as measured in the probe trial of the MWM (mean ± SEM; Control n=6; TDS n=12).

**Time Spent in Target Zone**

T-tests adjusted for unequal variances showed that there were no statistically significant differences in the means of percentage time spent in target zone between the two groups (figure 6.29).

Figure 6.29 Effect of TDS stress in Sprague-Dawley rats on percentage time in target zone as measured in the probe trial of the MWM (mean ± SEM; Control n=6; TDS n=12).
**Target Zone Entry Latency**

T-tests adjusted for unequal variances showed that there were no statistically significant differences in the means of target zone entry latency between the two groups (figure 6.30).

![Figure 6.30 Effect of TDS stress in Sprague-Dawley rats on target zone entry latency as measured in the probe trial of the MWM (mean ± SEM; Control n=6; TDS n=12).](image)

**Platform Crossings**

T-tests adjusted for unequal variances showed that there were no statistically significant differences in the means of platform crossings between the two groups (figure 6.31).

![Figure 6.31 Effect of TDS stress in Sprague-Dawley rats on the number of platform crossings as measured in the probe trial of the MWM (mean ± SEM; Control n=6; TDS n=12).](image)
**Swim Speed**

T-tests adjusted for unequal variances showed that there were no statistically significant differences in swim speed between the two groups (figure 6.32).

![Swim Speed Graph](image)

*Figure 6.32 Effect of TDS stress in Sprague-Dawley rats on swim speed as measured in the probe trial of the MWM (mean ± SEM; Control n=6; TDS n=12).*

### 6.3.1.3 Cued Trial

**Escape Latency**

T-tests adjusted for unequal variances showed that there were no statistically significant differences in the means of escape latency time between the two groups (figure 6.33).

![Escape Latency Graph](image)

*Figure 5.33 Effect of TDS stress in Sprague-Dawley rats on escape latency time as measured in the cued trial of the MWM (mean ± SEM; Control n=6; TDS n=12).*
6.3.2 The Elevated Plus Maze

TDS stress exposed and control Sprague-Dawley rats were moved in their home cages to the room adjacent to the experimental room and allowed to acclimatise for 1 hour. Rats were not exposed to any injections. The parameters measured were the same as those in the pharmacological validation study.

6.3.2.1 Anxiety-Related Behaviour

Open Arm Entries

T-tests revealed no statistically significant difference in mean percentage ratio open arm entries between Control and TDS groups (figure 6.34).

![Figure 6.34](image)

*Figure 6.34 Effect of TDS stress in Sprague-Dawley rats on ratio open arm entries in the EPM (mean ± SEM; Control n=11; TDS n=12).*

Open Arm Time

T-tests revealed no statistically significant difference in mean percentage ratio open arm time between Control and TDS groups (figure 6.35).
Figure 6.35 Effect of TDS stress in Sprague-Dawley rats on ratio open arm time in the EPM (mean ± SEM; Control n=11; TDS n=12).

Open Arm Latency

T-tests revealed no statistically significant difference in mean open arm latency between Control and TDS groups (figure 6.36).

Figure 6.36 Effect of TDS stress in Sprague-Dawley rats on open arm latency in the EPM (mean ± SEM; Control n=11; TDS n=12).

6.3.2.2 Prevalence of Extreme Behavioural Response

What is thus far apparent, is that TDS stress failed to evoke a marked effect on firstly cognitive behaviour (section 6.3.1) and now on aversive behaviour as predicted from earlier studies in our laboratory (Harvey et al., 2003; Naciti, 2002). However, behavioural variation and vulnerability to stress in both humans and animals is
increasingly being realised, such that in a given population only a certain percentage of individuals will react adversely or in a mal-adaptive manner to a stressor and go on to develop a psychiatric illness (Bale, 2006; Cohen and Zohar, 2004; De Kloet et al., 2005; Ellis et al., 2006). Indeed, the prevalence of PTSD in populations exposed to trauma has been shown to be only about 9-30 %, depending on the particular trauma (Breslau et al., 1998; Kessler et al., 1995). Bearing this important clinical observation in mind and in order to further investigate the unexpected lack of TDS stress on aversive behaviour, the EPM data were analysed further.

Cut-off behavioural criteria in the EPM based on those published by Cohen et al. (2003) were used to examine the prevalence of extreme behavioural responses. Rats were classified as well-adapted (0-60 seconds in closed arms and ≥ 8 open arm entries) or mal-adapted (0 open arm time ratio and no open arm entries).

As can be seen in table 6.1, no control rats made 8 or more open arm entries and two control rats made zero open arm entries. In the TDS group, 3 rats made 8 or more open arm entries and two rats made zero open arm entries. Table 6.2 shows that only one control rat spent 60 seconds or less in the closed arms, while none of the TDS rats spent less than 60s in the closed arms. Finally, as is evident from table 6.3, 2 control- and 2 TDS rats had an open arm time ratio of 0.

Table 6.1 Number of open arm entries made by Sprague-Dawley control and TDS rats (≥ 8 and 0 open arm entries highlighted).

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Table 6.2 Closed arm time of Sprague-Dawley control and TDS rats (≤ 60 seconds highlighted).

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Table 6.3 Ratio open arm time of Sprague-Dawley control and TDS rats (open arm ratio of 0 highlighted).

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<td>0.863797</td>
<td>9.514482</td>
</tr>
<tr>
<td>9</td>
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<td>5.320045</td>
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<tr>
<td>10</td>
<td>0</td>
<td>30.87892</td>
</tr>
<tr>
<td>11</td>
<td>1.920085</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>20.46276</td>
</tr>
</tbody>
</table>

Thus, if the specified open arm entry- and closed arm time criteria are taken into account, no well-adapted rats could be identified in either the control or TDS groups. Using the specified open arm entry- and open arm time ratio criteria, 2 control rats (18.18 %) and 2 TDS rats (16.7 %) could be classified as mal-adapted.
6.3.2.3 *Locomotor Activity*

**Closed Entries**

T-tests revealed no statistically significant difference in mean closed arm entries between Control and TDS groups (figure 6.37).

![Bar chart showing closed arm entries for Control and TDS groups.](image)

*Figure 6.37 Effect of TDS stress in Sprague-Dawley rats on closed arm entries in the EPM (mean ± SEM; Control n=11; TDS n=12).*

**Distance and Moving Time**

T-tests revealed no significant differences in mean distance or moving time between control and TDS groups (data not shown).

6.3.3 *The Acoustic Startle Response*

TDS stress exposed and control Sprague-Dawley rats were kept in their home cages and were moved individually to the experimental room immediately prior to testing. Rats were not exposed to any injections. The parameters measured were the same as those in the parametric and pharmacological validation studies.
6.3.3.1 Startle Amplitude

Maximum Startle Over Individual Trials

A two-way ANOVA applied to maximum startle ($V_{\text{max}}$) with trials as a repeated measure showed that there was no significant trial-by-group interaction, but that there was a significant trial effect over the two groups [$F(5.802; 104.430)=2.560, p=0.0250$]. Finally, T-tests adjusted for unequal variances performed for each trial showed that there were no statistically significant differences in $V_{\text{max}}$ between groups (figure 6.38).

![Figure 6.38 Effects of TDS stress in Sprague-Dawley rats on maximum startle over individual trials (mean ± SEM; n=12; Control ■; TDS ○).](image)

Baseline Startle

T-tests showed that there were no statistically significant differences in the means of the baseline startle (first 5 trials) between the two groups (figure 6.39).
Figure 6.39 Effect of TDS stress in Sprague-Dawley rats on baseline startle (mean ± SEM; n=12).

**Average Total Startle**

T-tests showed that there were no statistically significant differences in the means of the total startle (over all 30 trials) between the two groups (figure 6.40).

Figure 6.40 Effect of TDS stress in Sprague-Dawley rats on total startle (mean ± SEM; n=12).

**6.3.3.2 Habituation of Startle**

A two-way ANOVA applied to mean maximum startle (\(V_{\text{max}}\)) with blocks as a repeated measure showed that there was no significant block-by-group interaction, but that there was a significant block effect over the two groups \(F(2.6009; 57.2188)=6.9852,\)
p=0.0008]. Finally, T-tests revealed no statistically significant differences in mean V max between groups at any block (figure 6.41).

Figure 6.41 Effects of TDS stress in Sprague-Dawley rats on mean maximum startle over 6 blocks (mean ± SEM; n=12; Control ■ ---; TDS o --).

T-tests also revealed no statistically significant differences in the mean percentage habituation of maximum startle from block 1 to 6 between the two groups (figure 6.42).

Figure 6.42 Effect of TDS stress in Sprague-Dawley rats on percentage habituation of maximum startle (mean ± SEM; n=12).
6.3.3.3 Prevalence of Extreme Behavioural Response

As reasoned with regard to the effects of TDS stress on aversive behaviour, ASR data were analysed further.

Previously published cut-off behavioural criteria in the ASR were used to identify mal-adapted (non-habituation of startle i.e. ≤ 0 % habituation) and well-adapted (normal habituation) rats (Cohen et al., 2004). These authors did not specify values of percentage habituation than qualify as normal, but a value of ≥ 50 % was set based upon data published for normal Sprague-Dawley rats (53-60 %) (Malone et al., 2006; Varty et al., 1999; Varty et al., 2000).

Using these criteria, 5 control- and 4 TDS rats were identified as mal-adapted, whereas no control rats and 2 TDS rats were identified as well-adapted (table 6.4).

Table 6.4 Habituation of maximum startle in Sprague-Dawley rats (≤ 0 % and ≥ 50 % highlighted).

<table>
<thead>
<tr>
<th>Rat</th>
<th>Control</th>
<th>TDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40.56292</td>
<td>29.79167</td>
</tr>
<tr>
<td>2</td>
<td>27.16708</td>
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</tr>
<tr>
<td>3</td>
<td>-22.7866</td>
<td>-62.256</td>
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<tr>
<td>4</td>
<td>-8.16797</td>
<td>2.212052</td>
</tr>
<tr>
<td>5</td>
<td>-25.2874</td>
<td>14.24292</td>
</tr>
<tr>
<td>6</td>
<td>-59.7852</td>
<td>-55.0694</td>
</tr>
<tr>
<td>7</td>
<td>-39.9197</td>
<td>13.79564</td>
</tr>
<tr>
<td>8</td>
<td>7.420653</td>
<td>33.20409</td>
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<tr>
<td>9</td>
<td>44.31676</td>
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<td>11</td>
<td>42.12667</td>
<td>-20.873</td>
</tr>
<tr>
<td>12</td>
<td>10.04743</td>
<td>-36.2282</td>
</tr>
</tbody>
</table>

Therefore, 41.7 % and 33.3 % of the control and TDS group, respectively, could be classified as mal-adapted, whereas 0 % and 16.7 % of the control and TDS groups, respectively, could be classified as well-adapted.
6.3.3.4 Pre-Pulse Inhibition of Startle

T-tests showed no statistically significant differences in the mean percentage pre-pulse inhibition (first 10 startle trials vs. 10 PPI trials) of maximum startle between the two groups (figure 6.43).

![Graph showing pre-pulse inhibition in control and TDS groups.](image)

Figure 6.43 Effect of TDS stress in Sprague-Dawley rats on pre-pulse inhibition of maximum startle (mean ± SEM; n=12).

6.3.3.5 Maximum Startle at Different Stimulus Intensities

A two-way ANOVA applied to maximum startle (V max), with stimulus intensity trials as a repeated measure showed that there was no significant trial-by-group interaction, but that there was a significant stimulus intensity effect over the two groups \[F(2.765; 60.838)=92.972, \ p<0.000001\]. Finally, T-tests adjusted for unequal variances performed for each stimulus intensity trial showed that there were no statistically significant differences in mean V max between the two groups at any stimulus intensity (figure 6.44).
Figure 6.44 Effects of TDS stress on maximum startle amplitude at different stimulus intensities in Sprague-Dawley rats (mean ± SEM; n=12; Control ■ ——; TDS ◦ ---).

6.3.4 Synopsis

The following section provides a summary and brief discussion of the results obtained in the Sprague-Dawley TDS stress studies.

6.3.4.1 MWM

In Sprague-Dawley rats, TDS stress had no statistically significant effect on spatial memory acquisition as measured by escape latency, ratio time in target zone and target zone entry latency during training sessions. TDS stress also did not significantly influence search strategy choice or swimming speed during training. TDS stress had no statistically significant influence on spatial memory consolidation as measured by percentage time in target zone, target zone entry latency or platform crossings in the probe trial. Finally, both control and TDS rats displayed similar swim speeds in the probe trial, as well as similar escape latency times in the cued trial. In summary, these data indicate a lack of marked effect on TDS stress on spatial memory acquisition and consolidation in Sprague-Dawley rats.
As was the case in the pharmacological validation study, neither control nor TDS Sprague-Dawley rats displayed target quadrant bias or spent significantly more than 25% of the total probe trial time in the target quadrant. These data point to the possibility that the rats either failed to learn the task (memory acquisition and/or consolidation), or solved the task (finding the platform) by employing a strategy other than place learning. Although the reason for the possible failure in learning the task or employment of non-place learning strategies is unknown, it might be related to possible pre-existing anxiety or other characteristic in rats used in the study (see chapter 9).

**6.3.4.1 EPM**

In Sprague-Dawley rats, TDS stress had no statistically significant effect on anxiety-like behaviour in the EPM as measured by ratio open arm entries, ratio open arm time and open arm latency. Finally, TDS had no effect on locomotor activity, as indicated by a lack of significant differences in closed arm entries, moving time and distance between TDS and control groups.

When the number of rats displaying “extreme” behavioural response (maladaptive vs. well-adaptive) was examined using EPM cut-off criteria based on those previously published by Cohen et al. (2003), it was found that none of the control rats were well-adapted and 2 control rats (18.18%) were mal-adapted. Similarly, in the TDS group, no rats displayed well-adaptive behaviour, and 2 (16.7%) rats were mal-adapted. The low prevalence of well-adapted rats in the control and TDS group and the high percentage of mal-adapted rats in the control group is in contrast with the percentages published by Cohen et al. (2003) for Sprague-Dawley rats. However, the prevalence of mal-adapted behaviour (16.7%) induced by TDS stress in the current study is similar to the prevalence induced by predator stress in the Cohen study (22%) (table 6.5), as well as the clinical prevalence of PTSD in trauma-exposed populations (Breslau et al., 1998; Kessler et al., 1995).
The prevalence of extreme behavioural response in the current study (Sprague-Dawley rats) compared to the study by Cohen et al. (2003).

<table>
<thead>
<tr>
<th></th>
<th>Control Current Study</th>
<th>Stress Cohen et al. 2003 (TDS)</th>
<th>Stress Cohen et al. 2003 (Predator)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well-adapted</td>
<td>0 %</td>
<td>80 %</td>
<td>0 %</td>
</tr>
<tr>
<td>Mal-adapted</td>
<td>18.18 %</td>
<td>1.3 %</td>
<td>16.7 %</td>
</tr>
</tbody>
</table>

The differences in the prevalence well-adaptive behaviour in the control and TDS groups and mal-adaptive behaviour in the control groups may be explained by methodological differences between the two studies. For example, in the current study rats were exposed to TDS stress (restraint, forced swim, halothane followed by forced-swim re-stress), whereas the Cohen study used exposure to a predator as the stressor. In this regard, different types of stressors are known to induce different behavioural response profiles (Blanchard et al., 2001). In addition, aversive behaviour in the EPM was analysed using an automated tracking procedure, whereas Cohen et al. (2003) used observers to review taped sessions and rate behaviour. Although these observers were blind to group allocation, some rater bias and human error cannot be excluded, whereas the automated system in the current study prevents these possibilities. It should be mentioned however, that in the current study, digital scoring of EPM parameters was confirmed manually (scoring of taped EPM sessions by the experimenter) as no marked differences in results using the two methods could be found (data not shown). Alternatively, possible pre-existing anxiety in rats used in the study may have influence the prevalence of extreme behavioural response (see chapter 9).

**6.3.4.3 ASR**

In Sprague-Dawley rats, there was no significant trial-by-group interaction, and indeed TDS stress failed to significantly affect maximum startle over the 30 individual trials. TDS stress also failed to significantly affect baseline or total startle amplitudes, although baseline startle and total startle tended to be higher in the TDS group compared to the
control group. TDS stress appeared to have no significant effect on mean maximum
startle over the 6 blocks. Indeed, TDS stress failed to have any statistically significant
effect on habituation as measured between block 1 and 6. The mean habituation of startle
observed for both control (3.709 %) and TDS (7.347 %) groups were, however, markedly
lower than what is normally reported in the literature for normal Sprague-Dawley rats
(Malone et al., 2006; Varty et al., 1999; Varty et al., 2000). This stark decrease in
habituation of startle in both control and TDS groups suggest possible pre-existing
anxiety in rats used in the study (see chapter 9). Finally, TDS stress failed to affect pre-
pulse inhibition and there was also no statistically significant difference in startle
amplitude at any stimulus intensity tested.

Rats were classified using cut-off criteria for the ASR based on those previously
published by Cohen et al. (2004) for Sprague-Dawley rats, i.e. non-habituation
(habituation of ≤ 0 %) for mal-adaptive behaviour and normal habituation for well-
adaptive behaviour. Although these authors did not specify habituation percentages for
normal habituation, we set a value of ≥ 50 % based upon values published for normal
Sprague-Dawley rats (Malone et al., 2006; Varty et al., 1999; Varty et al., 2000).

When the number of rats displaying “extreme” behavioural response (maladaptive vs.
well-adaptive) was examined using these criteria, it was found that none of the control
rats were well-adapted and 5 (41.7 %) control rats were mal-adapted. In the TDS group, 2
(16.7 %) rats displayed well-adaptive behaviour, and 4 (33.3 %) displayed mal-adaptive
behaviour. In the control group, the prevalence of well-adaptive behaviour was lower and
the prevalence of mal-adaptive behaviour higher than those found in the Cohen study. In
the TDS group, the prevalence of both well-adapted and mal-adapted rats was slightly
higher than those found in the Cohen predator stress group (table 6.6).
Table 6.6 The prevalence of well-adaptive- and mal-adaptive behaviour in the current study (Sprague-Dawley rats) compared to the study by Cohen et al. (2004).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Current Study</td>
<td>Cohen et al. 2004</td>
</tr>
<tr>
<td>Well-adapted</td>
<td>0 %</td>
<td>20 %</td>
</tr>
<tr>
<td>Mal-adapted</td>
<td>41.7 %</td>
<td>5 %</td>
</tr>
</tbody>
</table>

The differences observed in the prevalence well-adaptive and mal-adaptive behaviour between the current study and the Cohen study may be explained by methodological differences between the two studies. As discussed for the EPM, the stressor type employed in the two studies differed, and this may influence the behavioural outcome. In addition, the ASR protocol used in the two studies may not have been similar, as the paper by Cohen and colleagues (2004) did not specify the exact protocol used. Finally, the Cohen study employed more strict criteria than the current study, subjecting rats to both the ASR and EPM and using cut-off criteria on data from both to classify rats. Alternatively, possible pre-existing anxiety in rats used in the study may have influence the prevalence of extreme behavioural response (see chapter 9).

6.4 TDS Stress Studies: Wistars

Having found no marked effect of TDS stress on MWM, EPM and ASR behaviour in Sprague-Dawley rats, the same series of experiments was conducted in Wistar rats, a strain reported to have an increased response to stress, as well as a more pronounced anxiety-like profile (Bekris et al., 2005; Rex et al., 2004; Staples and McGregor, 2006). Thus, the aim of the study was to determine is TDS stress was able to influence spatial learning and memory, aversive behaviour and arousal in a more stress or anxiety sensitive strain, by using validated animal tests (MWM, EPM and ASR).
6.4.1 The Morris Water Maze

TDS stress exposed and control Wistar rats were moved in their home cages to the room adjacent to the pool room and allowed to acclimatise for 1 hour. Rats were not exposed to any injections. The parameters measured were the same as those in the pharmacological validation study.

6.4.1.1 Acquisition Training

Escape Latency

A two-way ANOVA applied to escape latency times with sessions as a repeated measure showed that there was no significant session-by-group interaction, but that there was a statistically significant session effect over the two groups \([F(2.783; 55.659)=44.733, p<0.000001]\). Finally, T-tests adjusted for unequal variances performed for each session showed that there were no statistically significant differences in mean escape latency times between groups (figure 6.45).

![Graph showing escape latency over sessions for control and TDS stress groups.]

Figure 6.45 Effects of TDS stress in Wistar rats on escape latency (per session) as measured in the MWM (mean ± SEM; Control ■ — n=10; TDS ○ — n=12).
**Ratio Time Spent in Target Zone**

A two-way ANOVA applied to ratio time spent in target zone with sessions as a repeated measure showed that there was no significant session-by-group interaction, but that there was a statistically significant session effect over the two groups \(F(1.915; 38.301)=16.692, p=0.000008\]. Finally, T-tests adjusted for unequal variances performed for each session showed that there was a statistically significant difference in mean ratio time spent in target zone between groups at session 1, with the TDS group spending more time in the target zone than the Control group \([t(19.316)=-2.403 (p=0.0265)]\) (figure 6.46).

![Figure 6.46](image)

*Figure 6.46 Effects of TDS stress in Wistar rats on ratio time in target zone (per session) as measured in the MWM (mean ± SEM; Control ■ — n=10; TDS ○ --- n=12).*

**Target Zone Entry Latency**

A two-way ANOVA applied to target zone entry latency times with sessions as a repeated measure showed that there was no significant session-by-group interaction, but that there was a significant session effect over the two groups \(F(2.093; 41.865)=15.530, p<0.000007\]. Finally, T-tests adjusted for unequal variances, performed for each session
showed that there were no statistically significant differences in mean target zone entry latency times between groups (figure 6.47).

Figure 6.47 Effects of TDS stress in Wistar rats on target zone entry latency (per session) as measured in the MWM (mean ± SEM; Control ■ — n=10; TDS o --- n=12).

Swim Speed

A two-way ANOVA applied to swim speeds with sessions as a repeated measure showed that there was no significant session-by-group interaction, but that there was a statistically significant trial effect over the two groups [F(2.020; 40.402)=35.223, p<0.000001]. Finally, T-tests adjusted for unequal variances were performed for each session and the difference in mean swim speeds between groups was statistically significant at session 2 [t(19.985)=2.935 (p=0.0082)], with the TDS group swimming slower than the Control group (figure 6.48).
Figure 6.48 Effects of TDS stress in Wistar rats on swim speed (per session) as measured in the MWM (mean ± SEM; Control ■ — n=10; TDS ○ --- n=12).

**Swim Strategy**

T-tests adjusted for unequal variances were performed for the percentage of training trials where each specific search strategy was used. There were no statistically significant differences for any swim strategy between control and TDS rats (figure 6.49).
Figure 6.49 Effects of TDS stress in Wistar rats on the frequency of search strategy type used over all 16 training trials of the MWM (mean ± SEM; Control n=10; TDS n=12).

6.4.1.2 Probe Trial

Time Spent in Target Quadrant

A one-way ANOVA revealed statistically significant differences across quadrants for both the Control [F(3; 32)=30.72, p<0.0001] and TDS [F(3; 44)= 5.797, p=0.002] groups. Post-hoc Dunnett’s tests showed that Control rats spent a significantly higher percentage of time (p<0.01) in the opposite, and lower percentage of time (p<0.01) in the right quadrant compared to the target quadrant (figure 6.50 A). TDS rats also spent a significantly lower percentage of time (p<0.05) in the right quadrant compared to the target quadrant (figure 6.50 B).
Figure 6.50 Quadrant preference of the Wistar Control (A) and TDS (B) groups, as measured in the probe trial of the MWM (mean ± SEM; Control n=9; TDS n=12; ** p<0.01 vs. Target; * p<0.05 vs. Target).

**Ratio Time Spent in Target Zone**

T-tests adjusted for unequal variances showed that there were no statistically significant differences in the means of time spent in target zone between the two groups (figure 6.51).

Figure 6.51 Effect of TDS stress in Wistar rats on percentage time in target zone as measured in the probe trial of the MWM (mean ± SEM; Control n=9; TDS n=12).
**Target Zone Entry Latency**

T-tests adjusted for unequal variances showed that there were no statistically significant differences in the means of target zone entry latency between the two groups, although the TDS rats showed a tendency to faster target zone entry latencies compared to controls (figure 6.52).

![Figure 6.52 Effect of TDS stress in Wistar rats on target zone entry latency as measured in the probe trial of the MWM (mean ± SEM; Control n=9; TDS n=12).](image)

**Platform Crossings**

T-tests adjusted for unequal variances showed that there were no statistically significant differences in the means of platform crossings between the two groups (figure 6.53).

![Figure 6.53 Effect of TDS stress in Wistar rats on the number of platform crossings as measured in the probe trial of the MWM (mean ± SEM; Control n=9; TDS n=12).](image)
Swim Speed

T-tests for unequal variances showed that there were no statistically significant differences in swim speed between the two groups (figure 6.54).

![Swim Speed Graph](image)

Figure 6.54 Effect of TDS stress in Wistar rats on swim speed as measured in the probe trial of the MWM (mean ± SEM; Control n=10; TDS n=12).

6.4.1.3 Cued Trial

Escape Latency

T-tests adjusted for unequal variances showed that there were no statistically significant differences in the means of escape latency time between the two groups, although the TDS group tended to escape onto the platform faster than the Control group (figure 6.55).

![Escape Latency Graph](image)

Figure 6.55 Effect of TDS stress in Wistar rats on escape latency as measured in the cued trial of the MWM (mean ± SEM; Control n=9; TDS n=12).
6.4.2 The Elevated Plus Maze

TDS stress exposed and control Wistar rats were moved in their home cages to the room adjacent to the experimental room and allowed to acclimatise for 1 hour. Rats were not exposed to any injections. The parameters measured were the same as those in the pharmacological validation study.

6.4.2.1 Anxiety-Related Behaviour

Open Arm Entries

T-tests revealed no statistically significant difference in mean percentage ratio open arm entries between Control and TDS groups, although there was a trend towards decreased open arm entry by TDS rats (figure 6.56).

![Figure 6.56 Effect of TDS stress in Wistar rats on ratio open arm entries in the EPM (mean ± SEM; n=12).](image)

Open Arm Time

T-tests revealed no statistically significant difference in mean percentage ratio open arm time between Control and TDS groups, although again there was a trend towards decreased time spent in open arms by TDS rats (figure 6.57).
Figure 6.57 Effect of TDS stress in Wistar rats on ratio open arm time in the EPM (mean ± SEM; n=12).

**Open Arm Latency**

T-tests revealed no statistically significant difference in mean open arm latency between Control and TDS groups (figure 6.58).

Figure 6.58 Effect of TDS stress in Wistar rats on open arm latency in the EPM (mean ± SEM; n=12).

### 6.4.2.2 Prevalence of Extreme Behavioural Response

Like the Sprague-Dawley study, data from the Wistar EPM study were analysed further.
Cut-off behavioural criteria in the EPM, based on those published by Cohen et al. (2003), were again used to examine the prevalence of extreme behavioural response. Rats were classified as well-adapted (0-60 seconds in closed arms and ≥ 8 open arm entries) or mal-adapted (0 open arm time ratio and no open arm entries).

As is evident from table 6.7, only one control rat made 8 or more open arm entries and two control rats made zero open arm entries. Similar results were obtained in the TDS group, where one rat made 8 or more open arm entries and two rats made zero open arm entries.

Table 6.7 Number of open arm entries made by Wistar control and TDS rats (≥ 8 and 0 open arm entries highlighted.

<table>
<thead>
<tr>
<th>Rat</th>
<th>Control</th>
<th>TDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
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</tr>
<tr>
<td>2</td>
<td>5</td>
<td>2</td>
</tr>
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<td>3</td>
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</tr>
<tr>
<td>12</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 6.8 shows that while one control rat spent 60 seconds or less in the closed arms, no TDS rats spent 60 seconds or less in the closed arms. In both control and TDS groups, 2 rats had an open arm time ratio of 0 (table 6.9).
Table 6.8 Closed arm time of Wistar control and TDS rats (≤60 seconds highlighted).

<table>
<thead>
<tr>
<th>Rat</th>
<th>Control</th>
<th>TDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>220.26</td>
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<td>173.8</td>
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<tr>
<td>12</td>
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<td>119.2</td>
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</tbody>
</table>

Table 6.9 Ratio open arm time of Wistar control and TDS rats (open arm ratio of 0 highlighted).

<table>
<thead>
<tr>
<th>Rat</th>
<th>Control</th>
<th>TDS</th>
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<td>0</td>
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</tr>
<tr>
<td>5</td>
<td>50.26807</td>
<td>17.85057</td>
</tr>
<tr>
<td>6</td>
<td>20.57517</td>
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<tr>
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<td>17.74134</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>3.975617</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>39.39086</td>
</tr>
</tbody>
</table>

Thus, if the specified open arm entry- and closed arm time criteria are taken into account, no well-adapted rats could be identified in either the control or TDS groups. When the open arm entry- and open arm time ratio criteria where taken into account, 2 control rats (16.7 %) and 2 TDS rats (16.7 %) could be classified as mal-adapted.
6.4.2.3 Locomotor Activity

Closed Entries

T-tests revealed no statistically significant difference in mean closed arm entries between Control and TDS groups (figure 6.59).

![Graph showing closed arm entries for Control and TDS groups](image)

*Figure 6.59 Effect of TDS stress in Wistar rats on closed arm entries in the EPM (mean ± SEM; n=12).*

Distance and Moving Time

T-tests revealed no significant difference in mean distance or moving time between control and TDS groups (data not shown).

6.4.3 The Acoustic Startle Response

TDS stress exposed and control Wistar rats were kept in their home cages and were moved individually to the experimental room immediately prior to testing. Rats were not exposed to any injections. The parameters measured were the same as those in the parametric and pharmacological validation studies.
6.4.3.1 Startle Amplitude

Maximum Startle Over Individual Trials

A two-way ANOVA applied to maximum startle (V max) with trials as a repeated measure showed that there was no significant trial-by-group interaction, but that there was a significant trial effect over the two groups \[ F(5.206; 93.706) = 3.539, p = 0.005 \]. Finally, T-tests adjusted for unequal variances performed for each trial showed that there were statistically significant differences in mean V max between groups only at trial 20 \[ t(13.482) = 2.294, (p = 0.03845) \], with the TDS rats showing a lower mean V max than control rats (figure 6.60).

![Graph showing the effects of TDS stress on startle amplitude](image)

*Figure 6.60 Effects of TDS stress in Wistar rats on maximum startle over individual trials (mean ± SEM; Control ■ — n=11; TDS o --- n=12).*
**Baseline Startle**

T-tests revealed no statistically significant differences in the means of the baseline startle (first 5 trials) between the two groups (figure 6.61).

![Graph showing baseline startle](image1)

*Figure 6.61 Effect of TDS stress in Wistar rats on baseline startle (mean ± SEM; Control n=11; TDS n=12).*

**Average Total Startle**

T-tests showed no statistically significant differences in the total startle (over all 30 trials) between the two groups (figure 6.62).

![Graph showing total startle](image2)

*Figure 6.62 Effect of TDS stress in Wistar rats on total startle (mean ± SEM; Control n=11; TDS n=12).*
6.4.3.2 Habituation of Startle

A two-way ANOVA applied to mean maximum startle (V max) with blocks as a repeated measure showed that there was neither a statistically significant block-by-group interaction, nor a statistically significant block effect over the two groups. Finally, T-tests revealed no statistically significant differences in mean V max between groups at any block (figure 6.63).

Figure 6.63 Effects of TDS stress in Wistar rats on maximum startle over 6 blocks (mean ± SEM; n=12; Control ■ ---; TDS o ---).

T-tests also revealed no statistically significant difference in the mean percentage habituation of maximum startle (from block 1 to 6) between the two groups (figure 6.64).

Figure 6.64 Effect of TDS stress in Wistar rats on percentage habituation of maximum startle mean ± SEM; n=12).
6.4.3.3 Prevalence of Extreme Behavioural Response

Like the Sprague-Dawley study, data from the Wistar ASR study were analysed further.

Rats were classified using previously published cut-off criteria for the ASR of non-habituation (habituation of ≤ 0 %) for mal-adaptive behaviour (Cohen and Zohar, 2004; Cohen et al., 2004 & 2006). These authors did not specify values of percentage habituation than qualify as normal, so a value of ≥ 35 % was set based upon data published for normal Wistar rats (Fujiwara et al., 2006; Lehman et al., 1999). Using these criteria, 3 control- and 3 TDS rats were identified as mal-adapted, whereas 2 control rats and 6 TDS rats were identified as well-adapted (table 6.10).

Table 6.10 Habituation of maximum startle in Wistar rats (≤ 0 % and ≥ 50 % highlighted).

<table>
<thead>
<tr>
<th>Rat</th>
<th>Control</th>
<th>TDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-33.6391</td>
<td>27.6283</td>
</tr>
<tr>
<td>2</td>
<td>28.65891</td>
<td>54.61487</td>
</tr>
<tr>
<td>3</td>
<td>27.62712</td>
<td>62.80193</td>
</tr>
<tr>
<td>4</td>
<td>76.49165</td>
<td>67.2849</td>
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<td>5</td>
<td>20.4244</td>
<td>57.69623</td>
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<td>6</td>
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<td>26.81403</td>
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<td>8</td>
<td>33.40174</td>
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<td>30.77333</td>
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<td>11</td>
<td>87.09381</td>
<td>-11.7026</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-23.4917</td>
</tr>
</tbody>
</table>

Therefore, employing 0 % and ≥ 35 % habituation as cut-off criteria, 27.3 % and 25 % of the control and TDS group, respectively, could be classified as mal-adapted, whereas 18.18 % and 50 % of the control and TDS groups, respectively, could be classified as well-adapted.
6.4.3.4 Pre-Pulse Inhibition of Startle

T-tests revealed no statistically significant differences in the mean percentage pre-pulse inhibition (first 10 startle trials vs. 10 PPI trials) of maximum startle between the two groups (figure 6.65).

Figure 6.65 Effect of TDS stress in Wistar rats on pre-pulse inhibition of maximum startle (mean ± SEM; Control n=11; TDS n=12).

6.4.3.5 Maximum Startle at Different Stimulus Intensities

A two-way ANOVA applied to maximum startle (V max) with stimulus intensity trials as a repeated measure showed that there was no statistically significant trial-by-group interaction, but that there was a significant stimulus intensity effect over the two groups [F(2.009; 42.183)=97.384, p<0.000001]. Finally, T-tests adjusted for unequal variances performed for each stimulus intensity trial showed that there were no statistically significant differences in V max between the two groups at any stimulus intensity (figure 6.66).
Figure 6.66 Effect of TDS stress in Wistar rats on maximum startle amplitude at different stimulus intensities in Wistar rats (mean ± SEM; Control ■ — n=12; TDS ○ — n=11).

6.4.4 Synopsis

The following section provides a summary and brief discussion of the results obtained in the Wistar TDS stress studies.

6.4.1 The MWM

In Wistar rats, TDS stress had no statistically significant effect on spatial memory acquisition as measured by escape latency or target zone entry latency in training sessions. TDS rats did, however, have a significantly higher ratio time in target zone compared to controls in session 1 indicating a slightly better initial memory acquisition, but there were no differences in the remaining three sessions. TDS stress also did not significantly influence search strategy choice during training. TDS stress had no statistically significant influence on spatial memory consolidation as measured by percentage time in target zone, target zone entry latency and platform crossings in the probe trial. Target zone entry latency however, did tend to be lower in the TDS group compared to control. Both control and TDS rats displayed similar swim speeds in training
sessions and the probe trial, except for session 2 where TDS rats swam significantly slower than control rats. Finally, there were no statistically significant differences in escape latency times between TDS and control rats in the cued trial, although TDS rats did tend to escape faster.

In summary, these data indicate that TDS stress failed to have a marked effect on spatial memory acquisition and consolidation in Wistar rats. The only statistically significant effect of TDS stress was observed in ratio time in target zone in session 1, indicating a slightly better initial memory acquisition of TDS rats compared to control. Although statistical significance was not reached, TDS rats also had a lower target zone entry latency compared to controls. These findings are in accordance with that of a recent study where chronic unpredictable stress enhanced spatial memory acquisition in some training days, while having a less pronounced effect on spatial memory consolidation (Gouirand and Matuszewich, 2005).

Similar to the results of the pharmacological validation- and Sprague-Dawley studies, neither control nor TDS Wistar rats displayed target quadrant bias or spent significantly more than 25% of the total probe trial time in the target quadrant. As discussed, these data point to the possibility that the rats either failed to learn the task (memory acquisition and/or consolidation), or solved the task (finding the platform) by employing a strategy other than place learning. Although the reason for the possible failure in learning the task or employment of non-place learning strategies is unknown, it might be related to possible pre-existing anxiety or other characteristic in rats used in the study (see chapter 9).

6.4.2 The EPM

In Wistar rats, TDS stress had no statistically significant effect on anxiety-like behaviour in the EPM as measured by ratio open arm entries, ratio open arm time and open arm latency, but ratio open arm entries and ratio open arm time tended to be lower in TDS rats.
When the number of rats displaying "extreme" response (maladaptive vs. well-adaptive) was examined using EPM cut-off criteria based on those previously published by Cohen et al. (2003), it was found that none of the control rats were well-adapted and 2 (16.7 %) rats were mal-adapted. The same results were obtained in the TDS group, with no rats displaying well-adaptive behaviour, and 2 (16.7 %) rats displaying mal-adaptive behaviour. Once again, the low prevalence of well-adaptive behaviour in both control and TDS groups are in stark contrast to the percentages published by Cohen et al. (2003) for Sprague-Dawley rats. Moreover, the 16.7 % mal-adaptive behaviour prevalence rate of our control group considerably exceeds their published rate of 1.3 %. However, the prevalence of mal-adaptive behaviour (16.7 %) induced by TDS stress in the current study is similar to the prevalence induced by predator stress in the Cohen study (22 %) (table 6.11), as well as the clinical prevalence of PTSD in trauma-exposed populations (Breslau et al., 1998; Kessler et al., 1995).

Table 6.11 The prevalence of well-adaptive- and mal-adaptive behaviour in the current study (Wistar rats) compared to the study by Cohen et al. (2003).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Current Study</td>
<td>Cohen et al., 2003</td>
</tr>
<tr>
<td>Well-adapted</td>
<td>0 %</td>
<td>80 %</td>
</tr>
<tr>
<td>Mal-adapted</td>
<td>16.7 %</td>
<td>1.3 %</td>
</tr>
</tbody>
</table>

Similar to the results from the Sprague-Dawley study, the differences in the prevalence well-adaptive behaviour in the control and TDS groups and mal-adaptive behaviour in the control groups may be explained by methodological differences between the studies. Firstly, we used Wistar rats, whereas the Cohen study used Sprague-Dawley rats. In this regard, these two strains have been shown to differ in anxiety related behaviour and stress response (Bekris et al., 2005; Rex et al., 2004; Staple and McGregor, 2006). As discussed, the differences in stressor type and the method of behavioural analysis may also contribute to the differences in extreme behavioural response prevalence. Alternatively,
possible pre-existing anxiety in rats used in the study may have influenced the prevalence of extreme behavioural response (see chapter 9).

6.4.3 The ASR

In Wistar rats, there was no significant trial-by-group interaction and TDS stress failed to significantly affect maximum startle over 29 of the individual trials. At trial 20 however, the TDS group did have statistically significant lower maximum startle amplitude compared to the control group. Furthermore, TDS stress failed to significantly affect baseline or total startle amplitudes, although baseline startle and total startle tended to be lower in TDS rats. Finally, TDS stress failed to affect pre-pulse inhibition and there was also no statistically significant difference in startle amplitude at any stimulus intensity tested. TDS stress appeared to have no significant effect on mean maximum startle over the 6 blocks. Indeed, TDS stress failed to have any statistically significant effect on habituation as measured between block 1 and 6. In contrast to Sprague-Dawley rats, however, there was no statistically significant block effect over the two groups, indicating a possible lack of startle habituation. This is supported by the mean habituation values observed for control (17.725 %) and TDS (27.918 %) groups, which are slightly lower than what is reported in the literature for normal Wistar rats (35 %) (Fujiwara et al., 2006; Lehrmann et al., 1999). The somewhat lower habituation of startle in both control and TDS groups suggest possible pre-existing anxiety in all rats used in the study (see chapter 9).

Rats were classified using cut-off criteria for the ASR based on those previously published by Cohen et al. (2004) for Sprague-Dawley rats, i.e. non-habituation (habituation of ≤ 0 %) for mal-adaptive behaviour and normal habituation for well-adaptive behaviour. Although these authors did not specify habituation percentages for normal habituation, we set a value of ≥ 35 % based upon values published for normal Wistar rats (Fujiwara et al., 2006; Lehrmann et al., 1999).

When the number of rats displaying "extreme" response (maladaptive vs. well-adaptive) was examined using these criteria, it was found that 2 (18.18 %) control rats were well-adapted and 3 (27.3 %) control rats were mal-adapted. In the TDS group, 6 (50 %) rats
displayed well-adaptive behaviour, and 3 (25 %) displayed mal-adaptive behaviour. In the control group, the prevalence of well-adaptive behaviour was similar to that found in the Cohen study. The prevalence of mal-adaptive behaviour, however, was higher in the control group of the current study than in the controls of the Cohen study. In the TDS group, the percentage well-adapted rats was higher than that reported by the Cohen study, but the percentage mal-adapted rats was identical (table 6.12).

*Table 6.12 The prevalence of well-adaptive- and mal-adaptive behaviour in the current study (Wistar rats) compared to the study by Cohen et al. (2004).*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Current Study</td>
<td>Cohen et al. 2004</td>
</tr>
<tr>
<td>Well-adapted</td>
<td>18.18 %</td>
<td>20 %</td>
</tr>
<tr>
<td>Mal-adapted</td>
<td>27.3 %</td>
<td>5 %</td>
</tr>
</tbody>
</table>

The differences observed in the prevalence of mal-adaptive behaviour in the control groups and in the prevalence of well- and mal-adaptive behaviour in the stressed groups may be explained by methodological differences. Firstly, the current study used Wistar rats, whereas the Cohen study used Sprague-Dawley rats and these two strains have been shown to differ in their ASR (Hince and Martin-Iverson, 2005). Furthermore, as discussed earlier, the stressor type and ASR protocol used between the studies were/may have been different. Finally, as mentioned, the Cohen study employed more strict criteria than the current study. Alternatively, possible pre-existing anxiety in rats used in the study may have influence the prevalence of extreme behavioural response (see chapter 9).
7.1 Introduction

The aim of the study was to determine the effect of time-dependent sensitisation (TDS) stress on hypothalamic-pituitary-adrenal (HPA) axis activity (as assessed by plasma corticosterone) in Sprague-Dawley and Wistar rats. Furthermore, the effect of behavioural testing and chronic fluoxetine treatment (daily for 3 weeks) on the plasma corticosterone levels of TDS and control groups were also investigated.

7.2 Behavioural Test Exposed Groups

There were no significant differences in plasma corticosterone levels between control and TDS stress exposed Sprague-Dawley or Wistar rats subjected to the Morris water maze (MWM), elevated plus maze (EPM) or acoustic startle response (ASR) (data not shown).

7.3 Test Naive Groups

To determine if the lack of TDS effect on plasma corticosterone was due to the stress of behavioural testing, we investigated the effects of TDS stress on plasma corticosterone in behavioural test naïve Sprague-Dawley and Wistar rats.

7.3.1 Sprague-Dawley Rats

In test naïve Sprague-Dawley rats, TDS stress resulted in a significant increase in plasma corticosterone levels (figure 7.1).
Figure 7.1 Effect of TDS stress on plasma corticosterone levels in behavioural test naive Sprague-Dawleys (mean ± SEM; n=6; * p<0.05 vs. Control).

7.3.2 Wistar Rats

In test naive Wistar rats, TDS stress had no statistically significant effect on plasma corticosterone levels, although there was a slight trend towards decreased HPA activity in TDS stress exposed rats (figure 7.2).

Figure 7.2 Effect of TDS stress on plasma corticosterone levels in behavioural test naive free Wistars (mean ± SEM; n=12).
7.4 Test Naive, Saline vs. Fluoxetine Groups

To determine if chronic selective serotonin re-uptake inhibitor (SSRI) treatment has any influence on the activity of TDS stress induced changes in the HPA axis, we measured the plasma corticosterone levels of test naive, control and TDS stress exposed Wistar rats that received intraperitoneal injections of either saline or fluoxetine (10 mg/kg) for 21 days.

Results from a one-way ANOVA indicated statistically significant differences across groups [F(3; 44)=8.668, p=0.0001). Bonferroni tests revealed that TDS stress induced a significant suppression of plasma corticosterone in saline treated rats, an effect that was prevented by chronic fluoxetine administration (figure 7.3).

![Figure 7.3 Effect of fluoxetine on TDS-induced suppression of plasma corticosterone levels in behavioural test naive Wistars (mean ± SEM; n=12; *** p<0.001 vs. Saline Control).](image)

7.5 Synopsis

In Sprague-Dawley and Wistar rats exposed to one of the behavioural tests (MWM, EPM or ASR), TDS stress failed to have a significant effect on HPA axis activity, as measured by plasma corticosterone levels (data not shown).
As all three tests are considered to be quite stressful, we chose to investigate HPA activity in control and TDS rats that were not exposed to any behavioural testing. In Sprague-Dawley test naive rats, TDS stress significantly increased plasma corticosterone levels compared to controls. In test naive Wistar rats on the other hand, TDS stress failed to significantly affect plasma corticosterone levels, although there was a slight trend toward decreased plasma corticosterone levels in TDS stress exposed rats.

Based on the reported increased stress sensitivity and anxiety-like profile of Wistars compared to Sprague-Dawleys (Bekris et al., 2005; Rex et al., 2004; Staples and McGregor, 2006), we chose to perform all further HPA axis and protein expression studies in this strain. Of particular importance is the finding that chronic treatment with the SSRI fluoxetine prevented the TDS stress induced suppression of plasma corticosterone in saline treated, test naive Wistar rats.

One important observation, however, is the high mean plasma corticosterone of control rats in all the studies. Levels of 250-400 ng/ml were a constant finding, which is considerably higher than the values typically reported in the literature (20-75 ng/ml) for normal Sprague-Dawley and Wistar rats at the time of sacrifice (2pm for test exposed rats and 10am for test naive rats) (for example Li et al., 2004a; Mantsch et al., 2007; Seal et al., 2004; Solberg et al., 2001). Although differences between laboratories can contribute to variation in results, it is unlikely that such a marked increase can be explained by methodological differences alone. Therefore, in support of the behavioural data, the plasma corticosterone values point to the possibility that control rats were exposed to some stress. However, although high, these levels are not unique as there are some reports in the literature of similar levels (250-300 ng/ml) in control animals used in stress studies (Jeeva, 2004; Uys et al., 2006b).
8.1 Introduction

The aim of the study was to determine the effects of time-dependent sensitisation (TDS) stress on the expression of total and/or phosphorylated protein kinase B (PKB or Akt), cyclic AMP response element binding protein (CREB), extracellular regulated kinase (ERK) 1/2, glycogen synthase kinase (GSK)-3α/β, BCL-2, BAX and neuronal nitric oxide synthase (nNOS) in the hippocampus and frontal cortex of Wistar rats, using standardised Western blot experiments. In addition, where necessary, the phosphorylation signal was normalised against the total expression to give an indication of the relative activation of the protein.

8.2 Western blot Set-Up Studies

8.2.1 Standardisation of Western blots

The Western blot conditions were standardised/optimised for each individual antibody, in terms of the amount of protein loaded, the concentrations and incubation times for primary and secondary antibodies, the blocking and washing conditions, as well as the developing conditions (section 5.4.3.3). Thereafter, the exact standardised conditions were used during every subsequent Western blot experiment.
8.2.2 Validation of Densitometric Analysis

The aim of this study was to determine whether the method of densitometric analysis used was valid, by constructing a protein concentration curve. The expression of nNOS protein was determined in a series of total protein concentrations (figure 8.1). Different amounts of total protein (5-35 µg) from a single hippocampal sample were loaded onto a gel, and the density of the nNOS bands obtained after Western blotting determined by densitometric analysis with the ChemiDoc XRS system with Quantity One® 1-D analysis software (Bio-Rad).

![Figure 8.1 A. Representative blot and density of nNOS bands as a function of total protein concentration loaded.](image)

The results in figure 8.1 confirm that the method of densitometric analysis used was able to accurately ($R^2 = 0.9502$) detect linear increases in specific protein expression as a function of the total protein concentration loaded. Thus the method was considered to be validated and subsequently used in all the Western blot experiments.
8.2.3 Sensitivity of Western blots

Before initiating studies on the effects of TDS stress on selected protein expression, it was important to establish that standardised experimental conditions and validated method of densitometric analysis would be responsive to challenges known to evoke changes in these proteins.

Apart from their important role in the treatment of post-traumatic stress disorder (PTSD), antidepressants have in recent years been found to influence select intracellular signalling pathways that are intimately linked to their pharmacological response, including the cAMP-CREB-, brain derived neurotrophic factor (BDNF)-phosphoinositide-3 kinase (PI3K)-Akt, GSK-3- and ERK pathways, as well as apoptosis- and nitric oxide (NO) signalling pathways (chapter 3). The aim of this study was therefore to determine if pharmacologically induced changes in the expression of specific proteins could be detected, using the standardised Western blotting conditions and validated densitometric analysis methods.

Wistar rats were injected (i.p.) with either saline or fluoxetine for 21 days, and sacrificed after the last injection on day 21. The expression of total and/or phosphorylated Akt, CREB, ERK1/2, GSK-3α/β, BCL-2, BAX and nNOS was subsequently determined in the hippocampus and frontal cortex.

8.2.3.1 Akt

Protein expression of total and phosphorylated Akt was studied by Western blot using antibodies specific for total Akt and Akt phosphorylated on threonine 308. Total Akt antibody detected one band and p-Akt antibody 2 bands, with bands detected at 60 kDa analysed densitometrically.

T-tests showed that chronic fluoxetine administration failed to affect hippocampal total Akt and p-Akt expression (figure 8.2 A & B), as well as frontal cortex total Akt expression (figure 8.2 C). The drug was however, able to significantly reduce frontal cortex expression p-Akt (p<0.01; figure 8.2 D). Furthermore, when the phosphorylation
signal was normalised against total protein expression, t-tests revealed that while chronic fluoxetine had no statistically significant effect on the hippocampal p-Akt : Akt ratio, it did significantly reduce the frontal cortex p-Akt : Akt ratio (p<0.05; table 8.1).

Figure 8.2 Representative blots and the effect of fluoxetine on expression of hippocampal total Akt (A), hippocampal phosphorylated Akt (B), frontal cortex total Akt (C), frontal cortex phosphorylated Akt (D) (mean±SEM; ** p<0.01 vs. Saline).
Table 8.1 Effect of fluoxetine on hippocampal and frontal cortex p-Akt : Akt (mean±SEM; Sal hipp n=11; Fluox hipp n=12; Sal front cort n=8; Fluox front cort n=12; *p<0.05 vs. Saline).

<table>
<thead>
<tr>
<th></th>
<th>Hippocampus</th>
<th>Frontal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Fluoxetine</td>
</tr>
<tr>
<td>phospho:total</td>
<td>100 ± 2.53</td>
<td>94.01 ± 8.20</td>
</tr>
</tbody>
</table>

8.2.3.2 CREB

Protein expression of total and phosphorylated CREB was studied by Western blot, using antibodies specific for total CREB and CREB phosphorylated on serine 133. The antibodies detected 2 bands at approximately 46 and 43 kDa, respectively, and the total density of both bands were used in data analyses.

T-tests revealed that chronic fluoxetine administration significantly increased hippocampal total CREB expression (p<0.01; figure 8.3 A), but failed to affect hippocampal p-CREB expression (figure 8.3 B), as well as frontal cortex total CREB and p-CREB expression (figure 8.3 C & D). Furthermore, when the phosphorylation signal was normalised against total protein expression, t-tests revealed that chronic fluoxetine significantly reduced the hippocampal p-CREB : CREB ratio (p<0.01), while having no statistically significant effect on the frontal cortex p-CREB : CREB ratio (table 8.2).
Figure 8.3 Representative blots and the effect of fluoxetine on expression of hippocampal total CREB (A), hippocampal phosphorylated CREB (B), frontal cortex total CREB (C), frontal cortex phosphorylated CREB (D) (mean±SEM; ** p<0.01 vs. Saline).

Table 8.2 Effect of fluoxetine on hippocampal and frontal cortex p-CREB : CREB (mean±SEM; Sal hipp n=9; Fluox hipp n=8; Sal front cort n=9; Fluox front cort n=9; ** p<0.01 vs. Saline.).

<table>
<thead>
<tr>
<th>Hippocampus</th>
<th>Saline</th>
<th>Fluoxetine</th>
<th>Frontal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>phospho:total</td>
<td>100 ± 6.15</td>
<td>68.59 ± 6.00**</td>
<td>100 ± 6.15</td>
</tr>
</tbody>
</table>

8.2.3.3 ERK1/2

Protein expression of total and phosphorylated ERK1/2 was studied by Western blot, using antibodies specific for total ERK1/2 and ERK1/2 phosphorylated on threonine 202 and tyrosine 204. The antibodies detected bands at 42 and 44 kDa, and the total density of both bands were used in data analyses.
T-tests revealed that in the hippocampus, chronic fluoxetine administration significantly decreased total ERK1/2 expression (p<0.01; figure 8.4 A), while having no effect on p-ERK1/2 expression (figure 8.4 B). In the frontal cortex, on the other hand, chronic fluoxetine administration significantly increased (p<0.001) and decreased (p<0.001) total ERK1/2 and p-ERK1/2 expression, respectively (figure 8.4 C & D). Furthermore, when the phosphorylation signal was normalised against total protein expression, t-tests revealed that chronic fluoxetine had no statistically significant effect on the hippocampal p-ERK1/2 : ERK1/2 ratio, but that it did significantly reduce this ratio in the frontal cortex (p<0.001; table 8.3).

Table 8.3 Effect of fluoxetine on hippocampal and frontal cortex p-ERK1/2 : ERK1/2 (mean±SEM; Sal hipp n=11; Fluox hipp n=12; Sal front cort n=12; Fluox front cort n=12; *** p<0.001 vs. Saline).

<table>
<thead>
<tr>
<th>Hippocampus</th>
<th>Saline</th>
<th>Fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td>phospho:total</td>
<td>100±4.13</td>
<td>104.59±4.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Frontal cortex</th>
<th>Saline</th>
<th>Fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td>phospho:total</td>
<td>100±1.64</td>
<td>64.72±4.53***</td>
</tr>
</tbody>
</table>

A. ![Image A]

B. ![Image B]
Figure 8.4 Representative blots and the effect of fluoxetine on expression of hippocampal total ERK1/2 (A), hippocampal phosphorylated ERK1/2 (B), frontal cortex total ERK1/2 (C), frontal cortex phosphorylated ERK1/2 (D) (mean±SEM; ** p<0.01 vs. Saline; *** p<0.001 vs. Saline).

8.2.3.4 GSK-3α/β

Protein expression of total and phosphorylated GSK-3α/β was studied by Western blot, using antibodies specific for total GSK-3α/β and GSK-3α/β phosphorylated on serine 21 and -9. The antibodies detected bands at 46 and 51 kDa, and the total density of both bands were used in data analyses.

T-tests revealed that in the hippocampus, chronic fluoxetine administration significantly decreased total GSK-3α/β expression (p<0.001; figure 8.5 A), but failed to have an effect on p-GSK-3α/β expression (figure 8.5 B). In the frontal cortex, on the other hand, chronic fluoxetine administration had no effect on total GSK-3α/β expression (figure 8.5 C), but lead to a significantly increased p-GSK-3α/β expression (p<0.01; figure 8.5 D). When the phosphorylation signal was normalised against total protein expression, t-tests revealed that chronic fluoxetine had no statistically significant effect on either the hippocampal or frontal cortex p-GSK-3α/β : GSK-3α/β ratios (table 8.4).
Figure 8.5 Representative blots and the effect of fluoxetine on expression of hippocampal total GSK-3α/β (A), hippocampal phosphorylated GSK-3α/β (B), frontal cortex total
GSK-3α/β (C), frontal cortex phosphorylated GSK-3α/β (D) (mean±SEM; ** p<0.01 vs. Saline; *** p<0.001 vs. Saline).

Table 8.4 Effect of fluoxetine on hippocampal and frontal cortex p-GSK-3α/β : GSK-3α/β (mean±SEM; Sal hippoc n=8; Fluox hipp n=9; Sal front cort n=11; Fluox front cort n=8).

<table>
<thead>
<tr>
<th>Hippocampus</th>
<th>Saline</th>
<th>Fluoxetine</th>
<th>Frontal cortex</th>
<th>Saline</th>
<th>Fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td>phospho:total</td>
<td>100 ± 11.75</td>
<td>115.54 ± 9.15</td>
<td>phospho:total</td>
<td>100 ± 4.71</td>
<td>104.70 ± 8.20</td>
</tr>
</tbody>
</table>

8.2.3.5 BCL-2 and BAX

Expression of total BCL-2 and BAX was studied by Western blot, using antibodies specific for the total proteins. The antibodies detected bands at 26 and 23 kDa, respectively, and these bands were analysed densitometrically.

T-tests revealed no statistically significant effect of chronic fluoxetine administration on hippocampal total BCL-2 and BAX expression (figure 8.6 A & B), nor on frontal cortex total BCL-2 expression (figure 8.6 C). The drug did however, significantly reduce total BAX expression in the frontal cortex (p<0.05; figure 8.6 D).

A.  

B.  

C.  

D.
Figure 8.6 Representative blots and the effect of fluoxetine on expression of hippocampal total BCL-2 (A) and BAX (B), and frontal cortex total BCL-2 (C) and BAX (D) (mean ± SEM; * p<0.05 vs. Saline).

8.2.3.6 NOS

Expression of total nNOS was studied by Western blot, using an antibody specific for the total protein. The antibody detected a single band at 155 kDa and this band was analysed densitometrically.

T-tests revealed that chronic fluoxetine administration significantly increased hippocampal (p<0.01; figure 8.7 A) and frontal cortex (p<0.001; figure 8.7 B) nNOS expression.
Together, the data from the pilot studies confirm that the standardised Western blot conditions and densitometric analysis methods were capable of detecting pharmacologically induced changes in the hippocampal and/or frontal cortex expression of most of the proteins tested.

8.2.3.7 Synopsis

Chronic fluoxetine administration resulted in some significant changes in the total expression, phosphorylation and/or relative activation (phospho : total ratio) of selected proteins in the hippocampus or frontal cortex, providing confidence for the subsequent TDS stress studies.
Fluoxetine had no significant effect on hippocampal total Akt or p-Akt, or on frontal cortex total Akt. The SSRI did, however, significantly decrease frontal cortex p-Akt expression (figure 8.2 D), as well as the p-Akt : Akt ratio (table 8.1) in this brain region. These findings were somewhat unexpected, given that some studies show chronic antidepressant treatment to increase BDNF mRNA or protein expression (De Foubert et al., 2004), while BDNF leads to activation of the PI3K-Akt pathway (Patapoutian and Reichardt, 2001). Nevertheless, as no studies have been published that measure Akt or p-Akt (thr308) in these brain regions after chronic fluoxetine treatment, there is no literature to either support or contradict the finding of decreased frontal cortex p-Akt and phospho : total ratio.

Chronic fluoxetine increased total CREB expression in the hippocampus (figure 8.3 A), while having no significant effect on frontal cortex CREB expression. The first result is in accordance with a study by Nibuya and colleagues (1996), who found that chronic fluoxetine significantly increased total CREB expression in the rat hippocampus. No effects of the drug were however, observed for frontal cortex p-CREB levels, which is in contrast to a recent in vivo study showing an increase in frontal cortex p-CREB after chronic fluoxetine treatment (Laifenfeld et al., 2005a). However, the lack of effect of fluoxetine on hippocampal p-CREB in the current study is in accordance with the results of the Laifenfeld study (2005a). While fluoxetine failed to alter p-CREB, chronic treatment with the PDE inhibitor, rolipram, was able to increase frontal cortex p-CREB (data not shown), which is in accordance with the published effects of the drug (Asanuma et al., 1996; Fujioka et al., 2004; Monti et al., 2006).

With regard to ERK1/2, chronic fluoxetine treatment resulted in a significant decrease in hippocampal total ERK1/2 expression (figure 8.4 A), while having no effect on hippocampal p-ERK1/2 levels. In the frontal cortex, fluoxetine significantly increased total ERK1/2 levels (figure 8.4 C), while significantly decreasing p-ERK1/2 levels (figure 8.4 D), as well as the phospho : total ratio (table 8.3). These results are in accordance with the published ability of chronic fluoxetine in causing long-lasting inhibition of p-ERK1/2 expression in the rodent prefrontal cortex and hippocampus (Fumagalli et al., 2005).
Fluoxetine significantly decreased hippocampal total GSK-3α/β expression levels (figure 8.5 A), but had no statistically significant effect on its hippocampal phosphorylation (ser 21 and 9) or hippocampal and frontal cortex phospho:total ratios. In the frontal cortex, the SSRI again had no effect on total GSK-3α/β expression, although it did significantly increase p-GSK-3α/β levels (figure 8.5 D), a finding that is in accordance with results from published in vivo studies (Gould et al., 2004; Kaidanovich-Beilin et al., 2004; Li et al., 2004c).

Fluoxetine had no statistically significant effect on BCL-2 expression in the hippocampus or frontal cortex, or BAX expression in the hippocampus, but did cause a significant reduction of frontal cortex BAX expression (figure 8.6 D). Although no in vivo studies regarding the effect of chronic fluoxetine treatment on these proteins have been published, one study with other antidepressants has described an increase in hippocampal mossy-fibre BCL-2 immunoreactivity (Xu et al., 2003), while lithium has been shown to increase BCL-2 expression and decrease BAX expression in the rat hippocampus and frontal cortex (Chen et al., 1999; Li et al., 2003a).

With regard to nNOS, chronic fluoxetine significantly increased the enzyme’s expression in both the hippocampus and frontal cortex (figure 8.7 A & B). Pre-clinical studies have reported antidepressant treatment to decrease hippocampal NOS activity (Wegener et al., 2003), although negative (Wegener et al., 2004) and contradictory (Suzuki et al., 2003) findings have also been reported. No studies have examined the expression levels of nNOS in the rat hippocampus or frontal cortex after chronic fluoxetine treatment and thus, the current finding of increased nNOS expression in response to chronic fluoxetine treatment represents a novel finding. However, congruent with the current data is that clinical studies have described an increase in plasma NO metabolite levels after chronic antidepressant treatment of patients with depression and healthy controls (Chrapko et al., 2006; Lara et al., 2003).

The fluoxetine challenge studies have provided interesting information regarding its effects on cellular plasticity, resilience and survival related proteins in rat brain, some congruent with the literature, some less so, while others represent novel results. Nevertheless, the results confirm the ability of the applied Western blotting technique to
detect changes in the hippocampal and/or frontal cortex expression, phosphorylation and/or relative activation (phospho : total ratio) of the chosen proteins following an external challenge.

### 8.3 TDS Stress Studies

The aim of the study was to determine the effects of TDS stress on the expression of total and/or phosphorylated Akt, CREB, ERK1/2, GSK-3α/β, BCL-2, BAX and nNOS in the hippocampus and frontal cortex of Wistar rats. The response to TDS stress was evaluated in Wistars as this strain has been shown to have a more pronounced anxiety-like profile and an increased response to stress compared to Sprague-Dawleys (Bekris et al., 2005; Rex et al., 2004; Staples and McGregor, 2006).

#### 8.3.1 Akt

T-tests revealed no statistically significant difference in hippocampal total Akt and p-Akt expression between Control and TDS groups (figure 8.8 A & B). TDS stress also failed to significantly affect frontal cortex total Akt and p-Akt expression (figure 8.8 C & D).

When the phosphorylation signal was normalised against total protein expression, t-tests showed that TDS stress induced a significant increase in the hippocampal p-Akt : Akt ratio (p<0.05), while having no effect on this ratio in the frontal cortex (table 8.5).
Figure 8.8 Representative blots and the effect of TDS stress on expression of hippocampal total Akt (A), hippocampal phosphorylated Akt (B), frontal cortex total Akt (C) and frontal cortex phosphorylated Akt (D) (mean±SEM).

Table 8.5 Effect of TDS stress on hippocampal and frontal cortex p-Akt : Akt (mean±SEM; Hipp contr n=11; Hipp TDS n=11; Front cort contr n=8; Front cort TDS n=8; *p<0.05 vs. Saline).

<table>
<thead>
<tr>
<th>Hippocampus</th>
<th>Control</th>
<th>TDS</th>
<th>Frontal cortex</th>
<th>Control</th>
<th>TDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>phospho:total</td>
<td>100 ± 2.53</td>
<td>106.38 ± 1.22*</td>
<td>phospho:total</td>
<td>100 ± 3.09</td>
<td>99.38 ± 1.54</td>
</tr>
</tbody>
</table>

8.3.2 CREB

T-tests revealed that TDS stress had no statistically significant effect on hippocampal or frontal cortex total CREB and p-CREB expression (figure 8.9 A - D).
Figure 8.9 Representative blots and the effect of TDS stress on expression of hippocampal total CREB (A), hippocampal phosphorylated CREB (B), frontal cortex total CREB (C) and frontal cortex phosphorylated CREB (D) (mean±SEM).
When the phosphorylation signal was normalised against total protein expression, t-tests showed that TDS stress also failed to have a significant effect on the p-CREB : CREB ratio in the hippocampus and the frontal cortex (table 8.6).

Table 8.6 Effect of TDS stress on hippocampal and frontal cortex p-CREB : CREB (mean± SEM; Hipp contr n=9; Hipp TDS n=9; Front cort contr n=9; Front cort TDS n=9).

<table>
<thead>
<tr>
<th>Hippocampus</th>
<th>Frontal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>phospho:total</td>
<td>100 ± 6.15</td>
</tr>
</tbody>
</table>

8.3.3 ERK1/2

T-tests revealed that TDS stress had no statistically significant effect on hippocampal or frontal cortex total ERK1/2 and p-ERK1/2 expression (figure 8.10 A - D).

When the phosphorylation signal was normalised against total protein expression, t-tests showed that TDS stress also failed to have a significant effect on the p-CREB : CREB ratio in the hippocampus and the frontal cortex (table 8.7).

Table 8.7 Effect of TDS stress on hippocampal and frontal cortex p-ERK1/2 : ERK1/2 (mean± SEM; Hipp contr n=11; Hipp TDS n=12; Front cort contr n=12; Front cort TDS n=12).

<table>
<thead>
<tr>
<th>Hippocampus</th>
<th>Frontal cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>phospho:total</td>
<td>100 ± 4.13</td>
</tr>
</tbody>
</table>
Figure 8.10 Representative blots and the effect of TDS stress on expression of hippocampal total ERK1/2 (A), hippocampal phosphorylated ERK1/2 (B), frontal cortex total ERK1/2 (C) and frontal cortex phosphorylated ERK1/2 (D) (mean±SEM).

8.3.4 GSK-3α/β
T-tests revealed that TDS stress had no statistically significant effect on hippocampal or frontal cortex total GSK-3αβ and p-GSK-3αβ expression (figure 8.11 A - D).

Figure 8.11 Representative blots and the effect of TDS stress on expression of hippocampal total GSK-3αβ (A), hippocampal phosphorylated GSK-3αβ (B), frontal cortex total GSK-3αβ (C) and frontal cortex phosphorylated GSK-3αβ (D) (mean±SEM).
When the phosphorylation signal was normalised against total protein expression, t-tests showed that TDS stress also failed to have a significant effect on the p-GSK-3αβ : GSK-3αβ ratio in the hippocampus and the frontal cortex (table 8.8).

Table 8.8 Effect of TDS stress on hippocampal and frontal cortex p-GSK-3αβ : GSK-3αβ (mean ± SEM; Hipp contr n=8; Hipp TDS n=11; Front cort contr n=11; Front cort TDS n=11).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TDS</th>
<th></th>
<th>Control</th>
<th>TDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td>phospho:total</td>
<td>100 ± 1.75</td>
<td>97.88 ± 8.73</td>
<td>phospho:total</td>
<td>100 ± 4.71</td>
</tr>
</tbody>
</table>

8.3.5 BCL-2 and BAX

T-tests revealed that TDS stress had no statistically significant effect on hippocampal or frontal cortex total BCL-2 (figure 8.12 A & C) or BAX expression (figure 8.12 B & D).
Figure 8.12 Representative blots and the effect of TDS stress on expression of hippocampal total BCL-2 (A) and BAX (B), and frontal cortex total BCL-2 (C) and BAX (D) (mean±SEM).

8.3.6 nNOS

T-tests revealed that TDS stress had no statistically significant effect on hippocampal or frontal cortex total nNOS expression (figure 8.13 A & B).
8.3.7 Synopsis

TDS stress failed to affect the expression of hippocampal and frontal cortex expression of Akt, p-Akt (Thr308), CREB, p-CREB (Ser133), ERK1/2, p-ERK1/2 (Thr202/Tyr204, GSK-3α/β, p-GSK-3α/β (Ser21/Ser9), BCL-2, BAX and nNOS. TDS stress also failed to affect the ratio of frontal cortex p-Akt: Akt, and the ratio of hippocampal and frontal cortex p-CREB : CREB, p-ERK1/2 : ERK1/2 and p- GSK-3α/β : GSK-3α/β.

TDS stress did, however, significantly increase the hippocampal p-Akt : Akt ratio, compared to controls. The phospho : total ratio is considered to be an indication of a protein’s relative activation and as Akt activation is associated with neuronal survival, this finding is contrary to what was expected. Stress often decreases the expression of BDNF (reviewed in Duman and Monteggia, 2006) and other neurotrophic factors (Alfonso et al., 2006; Heine et al., 2005; Ueyama et al., 1997) in the hippocampus (section 3.2.2.1). This would be expected to lead to a decrease in receptor tyrosine kinase (RTK)-PI3K signalling and Akt phosphorylation and activation (section 3.3.2.3). However, some studies show an increase in hippocampal neurotrophic factor levels after stress and it has been suggested that this may reflect some compensatory mechanism (Faure et al., 2006). Thus, the increased hippocampal p-Akt : Akt ratio observed in the current study may be an indication of a compensatory upregulation of neurotrophic factors as a result of exposure to TDS stress.
The selection of proteins tested is based on their putative involvement in the pathophysiology and treatment of stress-related disorders based on postmortem, clinical and preclinical studies (chapter 4). The overwhelming lack of effect of TDS stress on the expression of these proteins, their phosphorylation and/or their relative activation (phospho : total ratio) was unexpected, considering the well-known effects of stress on neuronal plasticity, cellular resilience/survival and neurogenesis (reviewed in Charney et al., 2004, Manji and Duman, 2001, Mirescu and Gould, 2006 and Radley and Morrison, 2005), processes in which all of the selected proteins are believed to participate (chapter 4).

The mostly negative data from the protein expression studies may therefore indicate a lack of the TDS model's robustness. Alternatively, methodological factors may have contributed to the negative results (see chapter 9).
9.1 Introduction

The behavioural methods for assessment of Morris water maze (MWM), elevated plus maze (EPM) and acoustic startle response (ASR) behaviour by digital analysis were successfully validated (section 6.2), while the conditions and methodology for specific protein expression analysis by Western blot were also standardised for the study (section 8.2). While the investigation into fluoxetine-induced changes in cellular plasticity or resilience proteins forms a vital part of the authenticity and interpretation of the time-dependent sensitisation (TDS) data, it has importance in its own right. However, as this investigation was a secondary aim of the study, it is discussed in detail in section 8.2.3.7. The effects of TDS stress was henceforth studied using these methods.

The TDS model, consisting of a single prolonged stress (SPS) + re-stress (RS), failed to affect behaviour (section 6.3), but did modify endocrine function - although in a bidirectional fashion (chapter 7). The model also had no marked effect on the expression,
phosphorylation and/or relative activation of plasticity or resilience related proteins in brain regions known to be sensitive to stress (section 8.3). The relevance of these findings will be discussed in terms of the validity of the TDS model.

9.2 Behavioural Studies

The TDS model failed to have a significant effect on cognition, anxiety or arousal in Sprague-Dawley and Wistar rats as measured in the MWM, EPM and ASR, respectively. The results from the MWM and EPM are in contrast to earlier studies in our laboratory that provided evidence of face validity for the TDS model with regard to its effect on cognition and anxiety-like behaviour (Harvey et al., 2003; Naciti, 2002). In addition, SPS + RS did not alter arousal as measured in the ASR, which was intended as an extension to the behavioural validation of the model.

The reasons for the failed replication and extension of face validity with regard to behaviour may be related to a lack of reliability and robustness of the model, which will now be discussed.

9.2.1 TDS Model Reliability

Previous studies in our laboratory found that SPS + RS induced deficits in spatial learning and memory, and anxiety-like behaviour using the MWM and EPM, respectively (Harvey et al., 2003; Naciti, 2002). Possible explanations for the failed replication of those initial MWM and EPM findings in the current study include differences in laboratory conditions in which the experiments were performed, as well as differences in the method of behavioural analysis.

As mentioned, since the initial TDS stress validation was performed, a new behavioural laboratory has been set up, including equipment for computerised collection and monitoring of behaviour. In the original validation studies, the behavioural analysis was performed manually by the experimenter by reviewing and scoring taped sessions of the MWM and EPM. In the current study, however, behavioural analysis was performed using advanced computer and visual monitoring software, thereby eliminating any
possible human error and experimenter bias in the parameter measurements and data collection. It should be mentioned however, that to ensure comparability of the two methods, digital analysis of EPM behaviour was compared with manual analysis of taped sessions, and a very close correlation was found (data not shown).

The current study was also performed in a different building than the original study. In this regard, it has been shown that different laboratories frequently obtain different results in behavioural tests, even when using the same equipment and protocol (Lewejoehann et al., 2006). These differences may be attributed to different laboratory and housing conditions, and even differences between the experimenters performing the studies (Lewejoehann et al., 2006; Wahlsten et al., 2003). It is important to emphasise, however, that it is incorrect to assume that failure to confirm the initial findings is associated with the degree of standardisation of housing and test conditions (Würbel, 2000). Indeed, a valid animal model should be reliable and importantly, generalisable across different environments, including different housing conditions and laboratories (Wahlsten et al., 2003). It has even been suggested that results should be considered preliminary until they have been confirmed by other investigators and in other laboratories (Van der Staay, 2006). Thus, our inability to reproduce the initial TDS stress induced spatial impairment and anxiety point to a lack of reliability and generalisibility of the TDS model, suggesting that the model may not be relevant (Van der Staay, 2006).

Considering the MWM, however, deficits in spatial memory following stress may not reflect face validity for PTSD. Although PTSD often presents with working memory deficits as measured in various tasks (Koso and Hansen, 2006; Van Praag, 2004), a recent study using a virtual MWM task found no significant difference in training or probe trial performance between post-traumatic stress disorder (PTSD) patients and controls (Astur et al., 2006). Functional magnetic resonance imaging (MRI) did, however, show that PTSD patients displayed diminished hippocampal activity during the task and that this lack of hippocampal activation predicted PTSD severity (Astur et al., 2006). Thus, the failure of TDS to impair MWM performance may not represent a lack of face validity, as a human, virtual version of the task also found no difference in MWM performance.

Regarding pre-clinical models, the initial findings in our laboratory of SPS + RS induced impaired spatial learning and memory are the only published findings of cognitive
impairment in animal models of PTSD. In contrast, other PTSD stress sensitisation models have found no effects on MWM or hole-board learning paradigms (Buwalda et al., 2005; Wang et al., 2006). It has been suggested that the initial findings of impaired cognition in the SPS + RS model may have resulted from conditioned fear (Stam et al., 2007b), as the MWM task represents a model of in-context re-traumatisation with regard to swimming, which formed a part of the SPS and RS (Cohen et al., 2004).

\section*{9.2.2 Model Robustness}

The failure to reproduce initial validation findings may be also be related to the robustness of the TDS model. Data from the behavioural and endocrine studies indicate that control rats used in the study may have been exposed to some form of low-grade but unrecognised stress. However, it is believed that all research animals are exposed to some form of stress as a result of their housing in unnatural conditions (Morgan and Tromborg, 2007). A robust animal model should be capable of inducing a stress response regardless, and that the TDS model may be less effective in such conditions is a factor worth considering.

\subsection*{9.2.2.1 MWM: Lack of Target Quadrant Bias}

In none of the MWM studies did any group of rats (saline, scopolamine, control or TDS) display target quadrant preference or spend significantly more than 25\% of the total probe trial time in the target quadrant. In fact, in the TDS stress studies, Wistar rats displayed a preference for the right and/or opposite quadrants. These data point to the possibility that all the rats used in the MWM studies, either failed to learn the task (memory acquisition and/or consolidation), or solved the task (finding the platform) by employing a strategy other than place learning.

Escape latency time, the parameter used most often to track spatial memory acquisition, showed a statistically significant decrease as a function of session in saline, control and TDS rats. An escape latency of 10-15 seconds is sometimes used as a guideline for
sufficient spatial memory acquisition (Naciti, 2002). In the pharmacological validation study, the mean escape latency time for saline Sprague-Dawley rats dropped from 70 seconds on trial 1 to 7 seconds on trial 16 (data not shown). In the TDS stress studies, the mean escape latency time dropped from 86.3 seconds on trial 1 to 16.3 seconds on trial 16 for Sprague-Dawley controls, and from 89.3 seconds on trial 1 to 20.5 seconds on trial 16 for Sprague-Dawley TDS rats (data not shown). In Wistar control and TDS rats, the mean escape latency time dropped from 90 seconds and 88.6 seconds on trial 1, to 25.6 seconds and 23.1 seconds on trial 16, respectively (data not shown).

Therefore, although rats did not attain ideal mean escape latencies in the TDS studies, the values did reach an acceptable level by the end of training, indicating spatial memory acquisition. Thus, insufficient acquisition can be ruled out as a cause of the lack of target quadrant bias. In addition, it has been suggested that training rats in the MWM to a level of performance below the ideal (i.e. escape latency slightly higher than 10-15 seconds) allows for the detection of subtle differences between groups in the probe trial (Kraemer et al., 1996). Therefore, the escape latency values of rats in the TDS studies (16.3 to 25.6 seconds) shows that rats were not overtrained, which could have masked subtle cognitive impairments.

Although it could not be confirmed that a failure of memory consolidation or retrieval was the reason behind the lack of target quadrant bias, it is a possibility given the relatively high plasma corticosterone levels observed for all rats over the course of the study (section 9.3). In this regard, high levels of corticosterone has been reported to impair spatial memory retrieval (D’Hooge and De Deyn, 2001) and to negatively effect the hippocampus (Sapolsky, 2000), a structure that plays an important role in the temporary consolidation and retrieval of memory (Knowlton and Fanselow, 1998; Riedel et al., 1999).

As alluded to earlier, the use of non-place learning strategies is an alternative explanation for the observed lack of target quadrant bias. It has been shown that even when deprived of extra-maze cues, escape latencies decrease during training to a constant level of about 12.5 seconds after 3 days of training (Baldi et al., 2003). In the same study, rats tested in the standard MWM (extra-maze cues available) showed a continuing decrease of escape latency to approximately 7 seconds after 5 days of training. In the probe trial, however,
only rats tested in the standard MWM displayed target quadrant preference (Baldi et al., 2003). Since none of the rats tested in the current project displayed target quadrant bias, it is possible that non-place learning strategies were used successfully to find the platform during training, resulting in the observed decreased escape latencies over time.

The hypothesis of non-place learning may be supported by swim path analysis, made possible in this study by computerised monitoring of performance in the MWM. In saline treated Sprague-Dawley rats, the swim path employed most frequently over the 16 training trials was self-orientation (30.208%). In the TDS studies, both control and TDS Sprague-Dawley rats displayed scanning (28.8 and 29.2%) most often. In the Wistar control group, self-orientation (31.9%) was the most frequently observed swim path, whereas Wistar TDS rats displayed scanning (28.1%) most frequently. As discussed earlier (section 5.3.2.4), the swim path categories can be arranged in order of increasing efficacy, namely thigmotaxis - circling - random searching - scanning - self-orientating - approaching target - direct find. Thus, no group of rats tested employed the two most effective strategies (approach target and direct find), with only saline treated Sprague-Dawleys (pharmacological validation study) and Wistar controls (TDS stress study) using the third most effective strategy (self-orientation) most frequently. Wistar TDS- and Sprague-Dawley control- and TDS rats relied on scanning in most of the trials. These data might indicate the use of an inferior (non-place learning) strategy.

The exact cause for possible lack of memory consolidation and/or the use of inferior (non-place learning) search strategies is unknown, but certain characteristics of the rats used in the studies may be involved. In this regard, like all albino strains, both Sprague-Dawley and Wistar rats have modest visual capabilities (Kraemer et al., 1996; Prusky et al., 2002). As a result the rats might have been unable to utilise extra-maze cues and instead, located the platform in the training trials by taxis or praxis strategies which do not rely as heavily on visual information. In addition, although strategies using olfactory cues can be ruled out (the pool was stirred between every trial to eliminate odour trails), search strategies using auditory cues cannot be excluded as a white noise generator was not used in the current study.

Alternatively, pre-existing low-grade levels of anxiety or stress in rats used in the study may also account for a lack of memory consolidation and/or use of inferior (non-place
learning) search strategies. The hippocampus, especially the CA3-region, plays an important role in memory and is crucial for the acquisition and consolidation of spatial memory in the MWM (Florian and Roulet, 2004). In this regard, pre-clinical studies have shown the hippocampus to be particularly sensitive and vulnerable to stress (reviewed in McEwen et al., 2002). As mentioned earlier, high corticosterone levels impair hippocampus-dependent forms of memory (reviewed in Diamond et al., 2004 and Sapolsky, 1999) and may therefore explain the lack of target quadrant bias. This hypothesis is supported by data from the endocrine studies (chapter 7), where control corticosterone levels were noted to be ≥ 250 ng/ml, which can be regarded as high for control, unstressed animals. In support of this, similarly high corticosterone levels (±300 ng/ml) in control rats were found in earlier TDS stress studies in our laboratory where aversive behaviour in the EPM could also not be confirmed (Jeeva, 2004). In contrast, corticosterone levels of control rats in the initial validation studies in our laboratory - where the TDS model was found to induce cognitive impairment (MWM) and anxiety-like behaviour (EPM) - were markedly lower (±15 ng/ml). Clearly, the association between glucocorticoid (cortisol and corticosterone) levels and learning and memory is complicated, with both abnormally low and high levels disrupting cognition (Sapolsky, 2003; Wagner et al., 2005; Wolf, 2003). Thus, a change in spatial memory may be less reliable criterion for face validity in an animal model of PTSD (Stam et al., 2007b).

9.2.2.2 EPM and ASR: Prevalence of Extreme Behaviour

In both Sprague-Dawley and Wistar rats, TDS stress failed to have a statistically significant effect on aversive behaviour and arousal, as measured in the EPM and ASR, respectively.

When the prevalence of extreme behavioural response (maladaptive vs. well-adaptive) in the EPM and ASR was examined using cut-off criteria (Cohen et al., 2003; Cohen et al., 2004), it was found that control groups generally displayed a prevalence of low well-adaptive- and high mal-adaptive behaviour. In the EPM study, TDS rats generally also had a low prevalence of well-adaptive behaviour, but a percentage of mal-adapted rats that was similar to those reported by Cohen et al. (2003), and congruent with the prevalence that is reported for PTSD (Breslau et al., 1998; Kessler et al., 1995), i.e. 9-
30%. In the ASR study, TDS rats had a similar (Sprague-Dawley) or higher (Wistar) prevalence of well-adapted behaviour to that described in the Cohen stress groups. Similar to the EPM study, the percentage mal-adapted rats in the TDS groups corresponded to that of the Cohen study and also with the range given for PTSD (Breslau et al., 1998; Kessler et al., 1995). As discussed in the synopsis of chapter 6, the observed differences in the prevalence of extreme behaviour may be related to methodological differences between the Cohen and current studies. Alternatively, these data, especially the low prevalence of well-adaptive behaviour in all the control groups and some of the TDS groups, could indicate that the rats were exposed to subtle, undisclosed stress. This hypothesis is supported by data from the MWM and the neuroendocrine studies (chapter 7). If the rats were indeed exposed to some form of uncontrolled stress during housing, it might explain why no notable behavioural effects of TDS stress were evident that allowed the behavioural separation of TDS and control animals. However, it should again be strongly emphasised at this point, that all research animals are exposed to some form of stress due to their abnormal housing (Morgan and Tromborg, 2007) and that a robust PTSD model could still be expected to induce sufficient stress to separate control and model-stress exposed rats (see next section). Indeed, TDS stress did in fact induce altered corticosterone responses vs. controls as described in earlier studies in TDS stress, suggesting that the animals remained stress responsive, although this may not have been sufficient to evoke noticeable behavioural changes.

9.2.3 TDS Model Robustness: Re-stress and Habituation

The behavioural (MWM, EPM, ASR) data discussed above, as well as the overall raised corticosterone levels in control animals described earlier suggest that all rats (control and TDS) may have been exposed to a stressor unrelated to the applied SPS + RS. If this was indeed the case, the effect of TDS stress on spatial learning and memory, anxiety-like behaviour and arousal may have been masked.

The source of this unintentional stress exposure of control rats - if any - is unknown, but it can be speculated that housing conditions may have contributed to such a situation. Rats were bred and housed in the North-West University’s animal research centre, which unfortunately, experienced some problems with noise and temperature control during the
course of the study. These events were unfortunately beyond the control of the investigator. In addition, control and TDS rats were not handled daily over the two week study period and as daily handling is recommended to habituate animals to the stress of being handled, nonhandling of rats may have lead to a stressful reaction on the day of testing (behavioural testing, sacrifice). It should be mentioned, however, that in an attempt to determine the replicability and reliability of the TDS model, rats were intentionally not exposed to daily handling in order to mimic conditions of the original TDS model validation studies, which demonstrated significant behavioural (MWM, EPM), endocrine and neurochemical effects (Harvey et al., 2003; Naciti, 2002). Furthermore, other stress studies where daily handling was also not performed have also been published (Long et al., 2007; Wu et al., 1999). Finally, it should be mentioned that when rats subjected to daily handling were tested in the MWM, there were no differences spatial learning or memory between control and TDS groups (data not shown), and was subsequently not investigated for EPM and ASR studies.

The potentially stressful conditions described above (housing conditions and nonhandling), although not ideal, were not excessive in nature. Therefore, in the experimenter's opinion these conditions would not have been capable of inducing such high stress levels in control animals so as to mask the effect of TDS stress. Indeed, this is corroborated by the fact that TDS markedly, although bidirectionally, influenced corticosterone levels vs. controls, suggesting stress responsiveness similar to other TDS stress studies (Harvey et al., 2003; Naciti, 2002; Uys et al., 2006a) and a hallmark of PTSD (Yehuda, 2006). Again, it should be emphasised that it is believed that all research animals, in this case rats, experience some form of stress due to their abnormal environment (housing cages, artificial light etc.) and forced proximity to humans (Morgan and Tromborg, 2007). The explicit purpose of control groups, therefore, is to control for such stressors, as well as other changes that are not part of the intended experimental manipulation. Thus, even if experimental animals are slightly stressed or anxious, a robust PTSD model should still be able to induce sufficient stress to induce observable behavioural differences between control and model-stress exposed rats. The lack of an effect for SPS + RS on behaviour vs. controls, therefore is suggestive of a lack in robustness of the TDS model to evoke reproducible stress-related bio-behavioural changes.
Another plausible explanation for a lack of robustness of the TDS model may be related to the re-stress that is employed 7 days after the initial single prolonged stress. The rational behind the use of a re-stress is that it serves as a reminder of the initial stress experience, thereby potentiating the sensitisation and development of stress-related neurochemical and behavioural changes (Yehuda and Antelman, 1993). The situation is intended to mimic the clinical condition, where PTSD patients are continuously exposed to reminders of the original trauma (Van der Kolk, 1994), and which has been suggested to underlie the temporal persistence of PTSD (Brewin and Holmes, 2003) or even to induce kindling-like progression in symptoms (Post and Weiss, 1998). However, except for repeated traumatic events, PTSD patients are generally exposed to reminders of the event and not to the original trauma itself. Thus, models of PTSD should re-expose animals to reminders of the initial stress, but not necessarily be an actual component of the original stress itself.

In this regard, situational, in-context reminders of an initial stressor has been shown to extend the behavioural effects of the initial stressor (Maier, 2001) and repeated reminders may even induce a progressive increase in the behavioural response over time (Pynoos et al., 1996). One such model involves an acute exposure to footshock followed by weekly situational reminders, where the rat is returned to the box where shock was administered, but not actually re-shocked (Li et al., 2006b; Louvart et al., 2005; Pynoos et al., 1996). In the TDS model, however, rats are re-exposed to a real stress (swim stress), not just a reminder. Furthermore, in TDS, swim stress as the RS also forms part of the original SPS and in this regard, re-exposure to the same (homotypic) stress in the same environment (in context) may induce habituation to the stress. In support of this hypothesis, repeated in-context restraint stress has been shown to induce habituation in terms of 5-HT sensitisation (Clement et al., 1998), corticosterone response (Grissom et al., 2007) and anxiety and depression-like behaviour (Gregus et al., 2005). Importantly, habituation in terms of serotonin (5-HT) sensitisation is already evident after the first day of re-stress (Clement et al., 1998). Habituation may be the result of the animal’s ability to predict the outcome of the in-context stress (Overmier and Murison, 2005), as one of the abovementioned studies showed that when repeated restraint was carried out in a novel room (out of context), habituation was decreased (Grissom et al., 2007). Although the TDS model only involves one re-stress and not repeated re-stress as the latter studies have applied, habituation to an in-context reminder needs serious consideration, especially
since it may evoke behavioural habituation instead of the intended sensitisation. It is thus pertinent to note that SPS without re-stress induces hyperarousal (Khan and Liberzon, 2004), contextual freezing (Imanaka et al., 2006; Iwamoto et al., 2007; Takahashi et al., 2006), anxiety (Imanaka et al., 2006) and analgesia (Imanaka et al., 2006) in rodents. Together, these data suggest that the re-stress may be superfluous, or as described above, counterproductive in the hands of some investigators. In this regard, previous studies in our laboratory have found that while significant hippocampal and/or frontal cortex changes in monoamine levels are evident 7 days after the SPS, little changes could be detected 7 days after the introduction of a re-stress (Jeeva, 2004).

### 9.3 Corticosterone

TDS stress failed to affect plasma corticosterone levels in rats exposed to behavioural testing. These results are in contrast to results of the original validation studies in our laboratory (Harvey et al., 2003; Naciü, 2002), where TDS stress significantly reduced plasma corticosterone levels in rats exposed to the MWM and EPM. The current results are, however, similar to another more recent TDS study in our laboratory that failed to detect any difference in plasma corticosterone levels between controls and TDS rats previously exposed to EPM testing (Jeeva, 2004). However, behavioural testing in itself may be stressful to the animals (Márquez et al., 2005; Námëstková et al., 2005) and therefore influence the activity of the hypothalamic-pituitary-adrenal (HPA) axis. For this reason, further endocrine studies focused on animals that were not exposed to any behavioural tests. In these test naive rats, TDS stress either significantly increased (Sprague-Dawleys), or did not significantly change (Wistars) plasma corticosterone levels. In the pharmacological study in Wistar rats receiving daily saline injections, however, TDS stress significantly decreased plasma corticosterone levels vs. controls, a response akin to previous TDS studies. More importantly, especially from a predictive validity point of view, this effect was prevented by chronic fluoxetine treatment. On the other hand, Uys et al. (2006a) describe an increase in corticosterone following a similar TDS stress procedure.

Although variable, data from the endocrine studies indicate the ability of the TDS model to induce changes in HPA axis activity. Since clinical studies have found decreased (Glover and Poland, 2002; Mason et al., 1986; Thaller et al., 1999; Yehuda et al., 2000)
cortisol in PTSD, these data suggest face validity for the model. However, increased (De Bellis et al., 1994; Maes et al., 1998), as well as normal (Baker et al., 1999; Rasmusson et al., 2001) cortisol levels in patients with PTSD compared to controls have also been described (section 2.5.5.1). Indeed, one criterion for animal models of PTSD is that the stressor used should induce biobehavioural alterations with the potential for bidirectional expression (Yehuda and Antelman, 1993). Thus, the findings of increased, normal and decreased plasma corticosterone obtained in this and earlier studies, suggest an ability to evoke bidirectional changes, indicative of noteworthy face validity for the TDS model.

The changes in plasma corticosterone levels induced by SPS + RS also suggests possible construct validity for the TDS model, as altered activity of the HPA axis has been suggested to contribute to the pathophysiology of stress-related disorders such as PTSD (Heim et al., 2000; Raison and Miller, 2003; Wagner et al., 2005) and depression (Watson and Mackin, 2006). Furthermore, the ability of fluoxetine to reverse SPS + RS induced suppression of plasma corticosterone in Wistar rats suggests predictive validity for the TDS model, as PTSD patients often respond successfully to chronic fluoxetine treatment (Connor et al., 1999; Martenyi et al., 2002; Van der Kolk et al., 1994). In the original validation study of the TDS model in our laboratory, although the effect of fluoxetine on SPS + RS induced suppression of plasma corticosterone was not studied, fluoxetine was found to significantly prevent SPS + RS induced cognitive impairment and anxiety-like behaviour (Harvey et al., 2004a; Naciti, 2002).

Despite the favourable results on construct and predictive validity of the TDS model in the neuroendocrine studies, the exact role of the HPA axis in the pathophysiology and successful selective serotonin re-uptake inhibitor (SSRI) treatment of PTSD remains unclear. In depression (Nickish et al., 2005) and PTSD (Vermetten et al., 2006), chronic treatment with citalopram or paroxetine has been shown to reduce HPA axis responsiveness. However, chronic SSRI treatment in PTSD resulted in a further reduction in basal cortisol levels (Vermetten et al., 2006). Another study has reported that citalopram treatment decreases, and sertraline treatment increases morning cortisol levels in patients with chronic PTSD, despite a successful reduction of PTSD symptoms in response to both drugs (Tucker et al., 2004a). In a further study by the latter group, similar results were obtained in comorbid depression and PTSD, where chronic paroxetine treatment failed to affect cortisol levels, despite inducing significant
improvement of symptoms (Tucker et al., 2004b). Together, the results from these clinical studies re-emphasise the lack of understanding regarding the exact role of cortisol in the pathophysiology and treatment of PTSD. While indeed valuable, studying construct and predictive validity of an animal model using neuroendocrine correlates alone needs to be viewed with caution.

9.4 Protein Expression

TDS stress failed to affect the hippocampal and frontal cortex total or phosphorylated expression of any of the proteins tested. TDS stress also had no effect on the hippocampal or frontal cortex phospho : total ratio of any of the proteins measured, except for hippocampal protein kinase B (PKB or Akt), where TDS significantly increased the p-Akt : Akt ratio. As discussed in chapter 8, the increase in the relative activation of hippocampal Akt may be an indication of a compensatory upregulation of neurotrophic factors as a result of exposure to TDS stress. That chronic fluoxetine altered this marker (section 8.2.3.1) is also evidence of the relevance of this protein and these data.

The lack of effect of TDS stress on the expression, phosphorylation and/or relative activation of the majority of the proteins tested may indicate a deficiency in the model’s robustness, especially in light of the prior drug challenge studies with fluoxetine or rolipram (data not shown), which demonstrated some well recognised responses for drugs with antidepressant efficacy on cyclic AMP response element binding protein (CREB) (Asanuma et al., 1996; Fujioka et al., 2004; Laifenfeld et al., 2005; Monti et al., 2006; Nibuya et al., 1996), extracellular regulated kinase (ERK)1/2 (Fumagalli et al., 2005), glycogen synthase kinase-3 (GSK-3) α/β (Gould et al., 2004), BAX (Chen et al., 1999) (section 8.2.3.7). Indeed, preclinical studies using specific stress or genetic models of depression or chronic stress paradigms have reported an effect on the expression or phosphorylation of CREB, Akt, ERK1/2, GSK-3, BCL-2, BAX and nitric oxide synthase (NOS) (see section 3.3.3 for references).
It has been argued earlier that even if rats were exposed to some form of low-grade stress, as suggested by the behavioural and endocrine studies, a robust stress model should still have been able to induce differences in the expression, phosphorylation or relative activation of key neuronal plasticity, resilience and survival proteins, especially since fluoxetine induced changes were observed under the same housing conditions. Thus, while TDS stress did evoke significant, although variable effects on HPA axis activity akin to PTSD, it may be that habituation to the re-stress and lack of the model’s robustness may be responsible for the lack of effect on certain key cellular plasticity and resilience proteins, foremost of which would be CREB and ERK1/2. However, this cannot be confirmed since SPS alone, i.e. TDS without RS, has not been investigated in this regard in this or any other laboratory. Moreover, except for NOS (Harvey et al., 2004b; Harvey et al., 2005; Oosthuizen, 2003), none of the tested proteins have to date been investigated in the hippocampus or frontal cortex in specific PTSD models (stress sensitisation).

Therefore, the lack of an observable effect of TDS stress on these proteins may in fact not be related to a lack of robustness of the model at all, but simply that stress sensitisation-based models may not affect these signalling proteins. Interestingly, SPS models without the re-stress has been shown to induce glutamatergic dependent increases in arousal (ASR) (Kahn and Liberon, 2004), as well as changes in hippocampal and frontal cortex levels of some monoamines (Jeeva, 2004). Since glutamatergic and monoamine pathways are implicated in kindling, stress and stress sensitisation (Harvey, 2006; Stam, 2007b; Vermetten and Bremner, 2002a; Vermetten and Bremner, 2002b), these studies provide at least indirect evidence that SPS evokes activity in these pathways. However, as mentioned earlier, when a re-stress was added to the SPS, most of the hippocampal and frontal cortex monoamine levels returned to those of controls (Jeeva, 2004), suggesting that habituation to the re-stress may have been the cause for a lack of effect on the signalling proteins tested in the current study.

Factors related to the methodology of the protein expression study may also be involved in the observed lack of TDS stress on proteins tested, including the brain region, cell type and subcellular fraction examined. In the current protein expression studies, the entire hippocampus and frontal cortex was studied. In this regard, stress-induced atrophy typically occurs only in the CA3 region of the hippocampus (Watanabe et al., 1992b).
whereas decreased neurogenesis is observed in the dentate gyrus (Gould et al., 1997). Although some protein expression studies of stress examine the entire hippocampus or frontal cortex (Lee et al., 2006a; Xu et al., 2006b), others only look at selected subregions like the CA3 or dentate gyrus of the hippocampus (Luo et al., 2004) and the prefrontal- or even medial prefrontal cortex (Kuipers et al., 2003; Meller et al., 2003; Qi et al., 2006). Indeed, it has been shown that stress may selectively effect protein expression (BDNF) or phosphorylation (p-CREB) in the dentate gyrus, while having no effect on the hippocampus proper (Grønli et al., 2006). Thus, the use of the entire hippocampus and frontal cortex (including the dorsal-, lateral-, medial- and prefrontal cortices) in the current protein expression studies may have masked subtle changes induced by TDS stress in selected subregions of these structures. It should be mentioned, however, that previous TDS studies in our laboratory using the same method of dissection have found significant changes in NOS activity (Harvey et al., 2004b; Oosthuizen, 2003), nitric oxide (NO) metabolite levels (Harvey et al., 2005) and 5-HT1A receptor density and affinity (Harvey et al., 2003; Naciti, 2002).

The subcellular location of proteins may also have an influence on the detection of changes in protein expression, phosphorylation and activation. Due to the multitude of different proteins investigated in the current study, whole cell extracts (membranes, cytoplasm and nucleus) were used in all the Western blot experiments. However, some of the proteins tested are capable of changing subcellular location after activation. For example, p-ERK1/2 may remain in the cytoplasm or translocate into the nucleus and is capable of phosphorylating targets in both (section 3.3.3.3). Similarly p-Akt is active in both the cytoplasm and nucleus (section 3.3.3.2). Thus, the use of whole cell extracts in the current Western blot studies may have masked subtle, but functional subcellular changes in protein expression, phosphorylation and/or relative activation. In this regard, stress has been shown to induce prefrontal cortex ERK1/2 hyperphosphorylation especially in the most distal parts of dendrites (Trentani et al., 2002), whereas chronic antidepressant treatment has been shown to differentially regulate ERK1/2 phosphorylation in the cytosol and nucleus (Fumagalli et al., 2005).

Finally, hippocampal and frontal cortex tissues consisting of both neuronal and non-neuronal cells were used in the current study. In this regard, many of the proteins tested have been suggested to have different and sometimes opposite functions in different cell
types. For example, NO synthesised from NOS may have different functions depending on the cell type in which it is released, such as NO from nNOS in neurones and NO from inducible NOS (iNOS) in glia (Bishop and Anderson, 2005). Since Western blotting cannot distinguish between neuronal and non-neuronal tissues, alterations in protein expression, phosphorylation and/or relative activation in specific cell types may be missed or misinterpreted.

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**Conclusions and Future Research**

The current study successfully set up validated behavioural testing (Morris water maze (MWM), elevated plus maze (EPM)) in rats, using computerised tracking equipment and analysis software. In addition, the acoustic startle response (ASR) test protocol was parametrically and pharmacologically validated, using an automated startle system. With regard to protein expression studies, the Western blotting conditions were successfully standardised/optimised for each protein tested and the method of densitometric analysis was validated. Furthermore, the capability of the technique to detect alterations in protein expression following external challenge was confirmed by means of a pharmacological challenge with the selective serotonin re-uptake inhibitor (SSRI) fluoxetine. The aim of these pilot and validation studies was to reassess the time-dependent sensitisation (TDS) stress model as a putative animal model of post-traumatic stress disorder (PTSD) using more stringent and more diverse validation criteria.

The current study failed to find any significant effect of TDS stress (single prolonged stress (SPS) + re-stress (RS)) on spatial learning and memory and anxiety-like behaviour and thus, was unable to replicate earlier findings where TDS stress significantly impaired MWM performance (spatial learning and memory) and increased EPM associated
aversive behaviour (anxiety) (Naciti, 2002). These data point to a lack of reliability and generalisability of the TDS model. Furthermore, the extended characterisation of the TDS model with the ASR showed that SPS + RS did not induce any significant changes in arousal. Data from the MWM, EPM and ASR studies also indicate a lack of robustness of SPS + RS, which may be due to habituation to the re-stress. Thus, the overall inability of the TDS model to evoke behavioural changes indicates that it may suffer from a lack of relevance, as stipulated by Yehuda and Antelman (1993).

Results from the endocrine studies however, concur with earlier studies that TDS stress significantly, although not consistently, alters plasma corticosterone levels. A variable response to TDS stress mimics the clinical picture in PTSD. This suggests that the TDS model may possess noteworthy face and construct validity with regard to endocrine response. Furthermore, the finding that chronic fluoxetine administration is able to prevent SPS + RS induced suppression of plasma corticosterone levels, suggests possible predictive validity for the TDS model.

With regard to the protein expression studies, SPS + RS stress failed to significantly alter the expression, phosphorylation or relative activation of most of the signalling proteins tested, although these negative findings may not necessarily be an indication of a lack of validity or robustness of the TDS model.

In summary, although the TDS model demonstrates face-, as well as possible construct- and predictive validity in terms of its effects on endocrine function, data from the behavioural studies suggest that the model lacks reliability and generalisibility and hence, relevance. The current study therefore serves to highlight the importance of thorough validation of any behavioural animal model, preferably by investigators other than those involved in the original studies (Van der Staay, 2006). This would ensure that a model does indeed possess reliability and generalisability and thereby prevent the ongoing utilisation of irrelevant models and the unnecessary sacrifice of research animals.

With regard to the TDS model, the SPS + RS paradigm may not be a relevant animal model of PTSD and it is recommended that the re-stress be omitted from future studies. Although not confirmed in the current study, other studies using an SPS procedure without the re-stress have found promising results in terms of behaviour, neurochemistry.
and protein expression (Imanaka et al., 2006; Iwamoto et al., 2007; Jeeva, 2004; Kahn and Liberzon, 2004; Takahashi et al., 2006). Alternatively, the RS protocol should be re-investigated with regard to the repeat swim stress, as it may evoke habituation instead of sensitisation, by using a situational reminder of the SPS.

Although all the behavioural protocols were stringently validated, the sensitivity of the MWM and EPM may be further increased to enable the detection of more subtle effects of stress. For example, reducing the number of trials and increasing the intertrial intervals has been suggested to increase the difficulty of the MWM task, thereby allowing the detection of subtle cognitive differences between control and experimental animals (Mandel et al., 1989). Similarly, the additional analysis of ethological (risk assessment) parameters may render the EPM more sensitive (Rodgers et al., 1997). Furthermore, identifying extreme behavioural responses in animals and comparing control animals to stress exposed well-adapted and stress exposed mal-adapted animals (as identified by behavioural cut-off criteria), may more closely mimic PTSD with regard to individual stress susceptibility. In the current study, data from the EPM and ASR was used to identify well-adapted and mal-adapted individual animals by using previously published cut-off criteria. This may not be ideal however, as behaviour in these and other tests may differ considerably between different laboratories and even in the same laboratory over time (Wahlsten et al., 2006). It is therefore recommended that each laboratory establish its own cut-off criteria. One way to accomplish this is to test a large group of control animals over time and using the mean value of the behavioural parameter obtained in this way to define cut-off criteria for well-adapted and mal-adaptive behaviour.

Finally, although not a primary objective of the current study, the Western blot set-up studies uncovered some novel effects of chronic fluoxetine administration on the hippocampal and/or frontal cortex expression of proteins putatively involved in neuronal plasticity, resilience and survival. Therefore, further examination of the molecular mechanism of the drug, as well as its effects in a valid animal model of PTSD is warranted.


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