An investigation into the role of noradrenergic receptors in conditioned fear: relevance for posttraumatic stress disorder

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“Not by might,
nor by power,
but *by My Spirit*,”
says the Lord.

Zech 4:6

Abstract

Posttraumatic stress disorder is a debilitating anxiety disorder that can develop in the aftermath of a traumatic or life-threatening event involving extreme horror, intense fear or bodily harm. The disorder is typified by a symptom triad consisting of re-experiencing, hyperarousal and avoidance symptoms. Approximately 15-25% of trauma-exposed individuals go on to develop PTSD, depending on the nature and severity of the trauma. Although dysfunctional adaptive responses exist in multiple neurobiological pathways in the disorder, e.g. glutamate, GABA, glucocortocoids and serotonin, the noradrenergic system is particularly prominent and represents an important pharmacological target in attempts at preventing the development of PTSD posttrauma. However, current literature shows opposing and conflicting results regarding the effect of selective noradrenergic agents in memory processing, and the effect of modulation of selective noradrenergic receptors are spread over diverse protocols and paradigms of learning and fear also employing different strains of animals.

Fear conditioning is a behavioural paradigm that uses associative learning to study the neural mechanisms underlying learning, memory and fear. It is useful in investigating the underpinnings of disorders associated with maladaptive fear responses. Performing fear conditioning experiments with the aim of applying it to an animal model of PTSD, and relating these behavioural responses to a defined neural mechanism, will assist both in the elucidation of the underlying pathology of the disease, as well as the development of more effective treatment. This project has set about to re-examine the diverse and complex role of noradrenergic receptors in the conditioned fear response with relevance to PTSD. To the best of my knowledge, this study represents the first attempt at studying a range of noradrenergic compounds with diverse actions and their ability to modify

conditioned fear in a single animal model. This work thus introduces greater consistency and comparative relevance not currently available in the literature, and will also provide much needed pre-clinical evidence in support of treatment strategies targeting the noradrenergic system in the prevention of PTSD posttrauma.

The first objective of this study was to set up and validate a passive avoidance fear conditioning protocol under our laboratory conditions using the Gemini™ Avoidance System. The noradrenergic system plays a prominent role in memory consolidation and fear conditioning, while administration of β-adrenergic blockers, such as propranolol, have been shown to abolish learning and fear conditioning in both humans and animals. Propranolol has also demonstrated clinical value in preventing the progression of acute traumatic stress syndrome immediately posttrauma to full-blown PTSD. To confer predictive validity to our model, the centrally active β-adrenergic antagonist, propranolol, and the non-centrally acting β-adrenergic antagonist, nadolol, were administered to Wistar rats after passive avoidance fear conditioning training in the Gemini™ Avoidance System. Wistar rats were used because of their recognised enhanced sensitivity to stress. Evidence from this pilot study confirmed that propranolol 10 mg/kg significantly inhibits the consolidation of learned fear in rats, whereas nadolol is ineffective. This effectively validated our protocol and the apparatus for further application in this study and also confirmed the importance of a central mechanism of action for β-adrenoceptor blockade in the possible application of these drugs in preventing the development of PTSD posttrauma.

The second objective of this study was to investigate the role of α_1-, α_2-, β_1-, and β_2-receptors in a conditioned fear passive avoidance paradigm. This was done in order to investigate how selective pharmacological modulation of these receptors may modify the conditioned fear response, and whether any of these receptor systems might exert opposing effects in passive fear conditioning. Various centrally

active noradrenergic agents were employed over a 3-tiered dose response design, including the α_1-antagonist, prazosin, the α_2-agonist, guanfacine, the α_2-antagonist, yohimbine, the β_1-antagonist, betaxolol and the β_2-antagonist ICI 118551. The effect of post-exposure administration of these drugs on conditioned fear was compared to that of propranolol 10 mg/kg. Selected doses of betaxolol (10 mg/kg) and ICI 118551 (1 mg/kg) attenuated fear conditioning to an extent comparable to propranolol, as did prazosin (0.1 mg/kg). Yohimbine tended to boster fear learning at all doses tested, albeit not significantly, while guanfacine did not produce any significant effect on memory retention at any of the doses studied. This latter observation was surprising since yohimbine tended to bolster fear conditioning while earlier studies indicate that α_2-agonism impairs conditioned fear.

Concluding, this study has conferred validity to our passive avoidance model and has provided greater insight into the separate roles of noradrenergic receptors in contextual conditioned fear learning. The study has provided supportive evidence for a key role for *both* β_1- and β_2-antagonism, as well as α_1-antagonism, in inhibiting fear memory consolidation and hence as viable secondary treatment options to prevent the development of PTSD posttrauma. However, further study is required to delineate the precise role of the α_2-receptor in this regard.

Keywords: PTSD, Contextual fear conditioning, Passive avoidance, Memory consolidation, Learned fear, Propranolol, Nadolol, Betaxolol, ICI 118551, Prazosin, Yohimbine, Guanfacine.

Opsomming

Posttraumatiese stressteurnis (PTS) is ‘n angsversteuring wat verlammende effekte op sy slagoffers het. Dit kan ontwikkel na afloop van enige traumatiese of lewensbedreigende gebeurtenis wat met afgryse, doodsangs of ernstige liggaamlike leed gepaardgaan. Hierdie steurnis word gekenmerk deur ‘n drieledige sindroom van simptome, wat herlewing, oormatige opwekking en vermydingsgedrag insluit. Afhangende van die aard en felheid van die trauma sal nagenoeg 15-25% van individue wat aan trauma blootgestel is, voortgaan om PTS te ontwikkel. Alhoewel gebrekkige aanpassingsresponse van verskeie neurobiologiese bane in die onderliggende patologie van hierdie steurnis geïmpliseer word, bv. glutamaat, GABA, glukokortikoïede en serotonien, is die noradrenergiese stelsel besonder prominent en verteenwoordig dit ‘n belangrike farmakologiese teiken in pogings om die ontwikkeling van PTS na 'n traumatiese gebeurlikheid te stuit. Huidige literatuur lewer egter bewyse van opponerende en teenstrydige resultate met betrekking tot die effek van selektiewe noradrenergiese agente in die prosessering van geheue, asook die effek van modulering van selektiewe noradrenergiese reseptore. Hierdie resultate is met 'n verskeidenheid van diverse protokolle en paradigmas van leer en vrees verkry en daar is van verskillende dierestamme gebruik gemaak.

Vreeskondisionering is ‘n gedragsparadigma wat assosiatiewe leerprosesse inspan om die neurale meganismes onderliggend aan leer, geheue en vrees te bestudeer. Dit is veral nuttig om die onderliggende meganismes van steurnisse wat met wanaangepaste vreesresponse geassosieer word, te ondersoek. Die uitvoer van vreeskondisionering, met die doel om dit toe te pas in ‘n dieremodel van PTS, en om dan hierdie gedrag in verband te bring met ‘n gedefinieerde neurale meganisme, mag waardevol wees in die toeligting van die patologie wat die

steurnis ten grondslag lê, asook in die ontwikkeling van meer effektiewe behandeling. Hierdie projek het ten doel gehad om die diverse en komplekse rol van noradrenergiese reseptore in die gekondisioneerde vreesrespons, met betrekking tot PTS, te herevalueer. Sover as wat my kennis strek, verteenwoordig hierdie studie die eerste poging om ‘n reeks van noradrenergiese agente met diverse werkingsmeganismes se vermoë om gekondisioneerde vrees in ‘n enkele dieremodel te modifiseer, te bestudeer. Hierdie studie bring dus meer konsekwente en vergelykbare relevansie ter tafel wat nie tans in die literatuur beskikbaar is nie, en sal broodnodige prekliniese bewyse verskaf vir behandelingstrategieë wat die noradrenergiese stelsel teiken in die voorkoming van PTS

Die eerste doelwit van hierdie studie was om binne die raamwerk van vreeskondisionering ‘n passiewe vermydingsprotokol vir toepassing in die GEMINI™ "Avoidance System", op te stel en te valideer vir gebruik onder toestande in ons laboratorium. Die noradrenergiese stelsel speel ‘n kritiese rol in die konsolidasie van geheue en in vreeskondisionering, terwyl die toediening van β-adrenergiese antagoniste, soos propranolol, leer en vreeskondisionering ophef in mense en diere. Propranolol het ook kliniese waarde gedemonstreer om die progressie van akute traumatiese stressindroom onmiddelik posttrauma na volskaalse PTS te voorkom. Om voorspellingsgeldigheid aan ons model te verleen, is die sentraalwerkende β-adrenergiese antagonis, propranolol, en die nie-sentraalwerkende β-adrenergiese antagonis, nadolol aan Wistar-rotte toegedien na passiewe vermydingsvreeskondisionering in die Gemini™ "Avoidance System". Wistar-rotte is gebruik weens hul bewese verhoogde sensitiwiteit teenoor stres. Ons bevindinge in hierdie loodsstudie het bevestig dat propranolol 10 mg/kg die konsolidasie van aangeleerde vrees in rotte betekenisvol onderdruk, en dat nadolol nie hierdie effek toon nie. Hierdeur is ons protokol en apparaat effektief gevalideer vir verdere toepassing in hierdie studie en die belang van ‘n sentrale meganisme vir β-blokkerende middels in vreeskondisionering en die moontlike toepassing van hierdie middels in die voorkoming van die ontwikkeling van PTS na die trauma, is bevestig.

Die tweede doelwit van hierdie studie was om die rolle van α_1-, α_2-, β_1-, en β_2-reseptore in 'n klassieke gekondisioneerde vreesvermydingsparadigma te ondersoek. Die doel hiervan was om te sien hoe selektiewe farmakologiese manipulering van hierdie reseptore moontlik die gekondisioneerde vreesrespons kan beïnvloed, en of enige van hierdie reseptore moontlik opponerende effekte in passiewe vreeskondisionering produseer. Verskeie sentraalwerkende noradrenergiese middels is toegedien in 'n drieledige dosisresponsontwerp, insluitend die α_1-antagonis, prasosien, die α_2-agonis, guanfasien, die α_2-antagonis, johimbien, die β_1-antagonis, betaksolol en die β_2-antagonis, ICI 118551. Die effek van toediening van hierdie geneesmiddels onmiddellik na blootstelling aan vreeskondisionering is vergelyk met dié van propranolol 10 mg/kg. Geselekteerde dossise van betaksolol (10 mg/kg) en ICI 118551 (10 mg/kg), asook prasosien (0.1 mg/kg) het vreeskondisionering opgehef soortgelyk aan die effek van propranolol. Johimbien het geneig om vreeskondisionering te bevorder by alle dossise wat getoets is, alhoewel dit nie statisties betekenisvol was nie, terwyl guanfasien geen betekenisvolle effek op geheueretensie getoon het by enige dosis wat bestudeer is nie. Hierdie waarneming was verrassend, siende dat johimbien geneig het om vreeskondisionering te versterk, en vorige studies veronderstel dat α_2-agoniste gekondisioneerde vrees moet ophef.

Ten slotte het hierdie studie geldigheid aan ons passiewe vermydingsmodel verleen en groter insigte in die afsonderlike rolle wat die noradrenergiese reseptore in kontekstuele gekondisioneerde vrees speel, gebied. Hierdie studie het ondersteunende bewyse gelewer vir 'n sleutelrol vir beide β_1- en β_2-antagonisme, asook α_1-antagonisme, in die inhibering van konsolidasie van vreesgeheue en gevolglik is ook bewys gelewer van hierdie geneesmiddels as lewensvatbare sekondêre behandelingsopsies om die ontwikkeling van PTS na die traumatiese voorval te voorkom. Verdere studie is egter nodig om die presiese rol van die α_2-reseptor in hierdie verband op te klaar.

Opsomming

Sleutelwoorde: PTS, Kontekstuele vreeskondisionering, Passiewe vermyding, Konsolidasie van geheue, Aangeleerde vrees, Propranolol, Nadolol, Betaksolol, ICI118551, Prasosien, Johimbien, Guanfasien

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"*No man is an island, entire of itself*" (John Donne, 1642)

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List of Abbreviations

ACTH	Adrenocorticotrophic hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
cAMP	Cyclic adenosine monophosphate
CBC	Cut-off behavioural criteria
CeA	Central Amygdala
CNS	Central nervous system
CR	Conditioned response
cRBF	Regional cerebral blood flow
CREB	cAMP-response element-binding protein
CRF	Corticotrophin releasing factor
CRs	Conditioned responses
CS	Conditioned stimulus
E-LTP	Early Long-term potentiation
ERK2	extracellular regulated kinase
GABA	γ-amino-butyric acid
HPA	Hypothalamic-pituitary-adrenal
IS-LH	Inescapable Shock-Learned Helplessness
LA	Lateral amygdala
LC	Locus coeruleus
L-LTP	Late Long-term potentiation
LTP	Long-term potentiation
MAOIs	Monoamine oxidase inhibitors

mPFC	Medial prefrontal cortex
MRI	Magnetic resonance imaging
NA	Noradrenaline
NMDA	N-Methyl-D-aspartate
NOS	Nitric oxide synthase
NTS	Nucleus of the solitary tract
PET	Positron emission tomogrophy
PFC	Prefrontal cortex
PGi	Nucleus paragigantocellularis
PKA	Protein kinase
PSS	Predator Scent Stress
PTSD	Posttraumatic Stress Disorder
SNRIs	serotonin norepinephrine reuptake inhibitors
SPECT	Single photon emission computed tomogrophy
SPS	Single prolonged stress
SSRIs	Selective serotonin reuptake inhibitors
STL	Step-through latency
TCAs	Tricyclic antidepressants
TDS	Time-dependent sensitisation
UR	Unconditioned response
US	Unconditioned stimulus
vmPFC	Ventromedial prefrontal cortex

CHAPTER 1 Introduction

1.1 PROBLEM STATEMENT

Posttraumatic stress disorder (PTSD) is an anxiety disorder that often develops after exposure to a traumatic or life-threatening event during which intense fear, horror or helplessness are experienced. It is a syndrome characterised by three symptom clusters, viz. re-experiencing (recurrent intrusive thoughts, nightmares, flashbacks etc.), avoidance/numbing behavior (avoiding thoughts, feelings and situations reminiscent of the trauma), and persistent hyperarousal (hypervigilance, sleep disturbances and increased startle response) (American Psychiatric Association, 2000). The general population shows a PTSD prevalence rate of approximately 7% (Kessler et al. 2005). The South African population are at particular risk of developing PTSD, with approximately 75% of South Africans experiencing at least one traumatic event in their lifetime (Kaminer et al. 2008), while Yehuda et al. (2009) found that approximately 15-25% of trauma-exposed individuals go on to develop PTSD, depending on the nature and severity of the trauma, as well as other predisposing risk factors. A significant proportion of the South African population can therefore be at risk of developing PTSD, and if left untreated, PTSD may become progressively worse, eventually leading to other anxiety disorders, depression and suicide (Johnsen 2002). In general, PTSD can be regarded a disorder of memory, characterised by reduced explicit memory function (Elzinga, Bremner 2002, Weber et al. 2005) but enhanced fear memory related to the traumatic event (Quirk et al. 2006, Rauch et al. 2006).

Current pharmacological treatment regimes for PTSD include various classes of antidepressants, such as the selective serotonin reuptake inhibitors (SSRIs), serotonin norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs). Despite this broad

armamentarium of drugs, for many patients treatment remains inadequate (Albucher, Liberzon 2002, Ravindran, Stein 2009, Schoenfeld et al. 2004). Moreover, the high degree of comorbid major depression in many PTSD patients further complicates treatment and compromises a favorable outcome (Ravindran, Stein 2009). Anti-adrenergic agents such as prazosin have been studied in the treatment of sleep-related disturbances in chronic PTSD (Raskind et al. 2003, Raskind et al. 2007, Taylor et al. 2008), while the use of β-adrenergic antagonists have been suggested as a treatment option in the secondary prevention of PTSD after the trauma (Cahill et al. 1994, Reist et al. 2001, Vaiva et al. 2003). With the growing awareness that PTSD involves a disturbance in excitatory vs inhibitory transmitters (MacKenzie et al. 2008), anticonvulsants have been proposed as an alternative treatment due to their putative anti-kindling effects. However, this is complicated by the observation that benzodiazepines, which amplify inhibitory γ-amino-butyric acid (GABA) transmission, may actually exacerbate PTSD symptoms (Gelpin et al, 1996). In general, however, many patients remain treatment resistant, and impeding the progression of acute traumatic stress syndrome immediately posttrauma to full-blown PTSD is thus imperative. However, in order to address this, a deeper knowledge of the underlying neurobiology of PTSD is necessary.

Functional neuroimaging studies and basic research has helped to identify three brain regions possibly involved in the pathology of PTSD, viz. the hippocampus, amygdala and medial prefrontal cortex (mPFC). Functional relationships between these three brain areas have also been established (Bremner et al. 2005, Shin et al. 2006).

The hippocampus is important in the regulation of the stress response and in the function of working/declarative memory (Bremner 1999, Elzinga, Bremner 2002), and has been found to be significantly smaller in PTSD patients (Lanius et al. 2006). This shrinkage of the hippocampus has been suggested to be responsible for the impairment in new learning in patients with PTSD, the inability to deal with intrusive memories (Bodnoff et al. 1995, Luine et al. 1994) as well as deficits in regulating the stress response. On the other hand,

memories related directly to the trauma become entrenched while extinction of these memories also does not occur so that the patient's daily life becomes incapacitated by the events of the original trauma. The amygdala is involved in the formation and retrieval of emotional and aversive memories (Shin et al. 2006) and is important in the extinction of learned fear (Meyers, Davis 2007). Research has provided evidence for amygdala hyperresponsivity in PTSD (Liberzon et al. 1999b, Hendler et al. 2003, Shin et al. 2004), as well as evidence for reduced amygdala volume in patients with PTSD (Rogers et al. 2009, Shin et al. 2004). Changes in neuronal plasticity in fear-related subdivisions of the amygdala also appear to be associated with trauma-induced social deficits in PTSD (Mikics et al. 2008). The prefrontal cortex (PFC) plays an important role in working memory function (Ramos, Arnsten 2007) and is also responsible for inhibiting the response of the amygdala to stress (Shin et al. 2004). PTSD has been associated with an overactive amygdala and impaired PFC function (Bremner 2002). On the other hand, the medial prefrontal cortex (mPFC) has a modulating role in emotional processes such as conditioned fear extinction (Quirk, Beer 2006) and exerts indirect inhibitory control over hypothalamic-pituitary-adrenal (HPA) axis responses (Radley et al. 2009). Indeed, PTSD has been associated with an exaggerated HPA axis negative feedback response (Kohda et al. 2007, Liberzon et al. 1999a, Liberzon et al. 1999b), which provides further evidence of impaired PFC functioning. It therefore seems that PTSD is characterised by alterations in PFC neural activity. This, together with the increase in noradrenergic activity leads to an impairment in the extinction of the fear response, also a typical characteristic of PTSD.

A meta-analysis of structural brain abnormalities in PTSD also concludes that PTSD is associated with significantly smaller prefrontal lobe volumes (Karl et al. 2006, Shin et al. 2004). It can thus be concluded that PTSD is accompanied by smaller volumes in multiple frontal lobe and limbic system structures.

The noradrenergic system plays a prominent role in memory consolidation and fear conditioning (Cahill et al. 2000, Nielson et al. 1999, Roozendaal et al.

1997, Roozendaal et al. 1997, Vaiva et al. 2003, van Stegeren 2008). There is also convincing evidence for enhanced noradrenergic activity in PTSD (Newport, Nemeroff 2000, Ravindran, Stein 2009), including increased urinary noradrenaline (NA) levels (De Bellis et al. 1994, Kosten et al. 1987, Yehuda et al. 1992) as well as elevated NA in the cerebrospinal fluid (Geracioti et al. 2001). Furthermore, adrenergic receptors in sufferers of chronic PTSD are hypersensitive (Morgan et al. 1993, Southwick et al. 1993), while platelet α_2-adrenergic receptors are reduced in PTSD patients (Perry et al. 1987), which in effect will amplify the response to already elevated noradrenaline levels. These data suggest a down-regulatory response subsequent to chronically elevated NA levels.

The noradrenergic system consists of 3 families of adrenergic receptors, namely the α_1-, α_2- and the $\beta_{1\text{-}3}$-receptors, with especially the $\alpha_{1/2}$- and the $\beta_{1/2}$-receptors significantly influencing the consolidation of emotional memories (Cahill et al. 1994), in particular where emotional learning induces hormone release. Adrenal hormones released after a traumatic event facilitate consolidation of emotional memory (fear memory) (Roozendaal 2002), while administration of β-adrenergic blockers, such as propranolol, have been shown to abolish learning and fear conditioning in both humans (Pitman et al. 2002) and animals (Cahill et al. 2000, Roozendaal et al. 1997). Indeed, activation of the amygdala in humans in response to emotional pictures is attenuated by propranolol, suggesting that the noradrenergic system acts to enhance fear acquisition and fear conditioning through direct activation of the amygdala. Furthermore, these data also demonstrate that noradrenergic antagonists can be used to inhibit fear conditioning and hence the neurodevelopment of PTSD following the trauma.

As discussed above, noradrenergic β-receptors are essential in emotional learning and memory of fear (Pitman et al. 2002, Roozendaal et al. 2004). However, while non-selective blockade of β-receptors have been found to repeatedly produce attenuated fear acquisition and fear conditioning in humans and animals, other studies suggest that targeting β_1- and β_2-receptors, like α_1-

and α_2-receptors, have opposing functional effects in tasks requiring memory and learning (Ramos et al. 2005) and in PTSD (Shalev et al. 2011), which may complicate the treatment of PTSD, especially if these drugs are to be used to prevent the development of the illness. This warrants investigation into the effects of selective β_1- and β_2-adrenergic blockade on fear conditioning and its application in an animal model of PTSD. Furthermore, recent studies by Cohen and colleagues (2010) showed that post-stressor administration of propranolol was ineffective in attenuating behavioural disruption in an animal model of PTSD, while Schneider et al (2011) found that propranolol was only effective in attenuating fear conditioning in a passive avoidance paradigm when combined with an additional stressor such as swim stress. Therefore, additional studies are not only necessary to address the role of propranolol in different paradigms of fear conditioning as well as its effectiveness in preventing the development of PTSD-like symptoms, but also to delineate the precise functional neurobiology of the different adrenergic receptors in fear conditioning.

Fear conditioning refers to the behavioural paradigm in which an animal learns to predict an aversive event (Maren 2001). A neutral conditioned stimulus (CS) is associated with an aversive unconditioned stimulus (US), and the animal learns to associate these stimuli and thus learns to fear the neutral stimulus as well, even in the absence of the aversive stimulus. Also called Pavlovian conditioning, it is useful in investigating and understanding the neural mechanisms and circuitry underlying learning and memory, especially fear memory. Avoidance is part of the symptom triad observed in PTSD, and is brought about by learning to fear a context or conditioned (neutral) stimulus by associating it with an unconditioned (aversive) stimulus. Because PTSD is a disorder of memory characterised by heightened fear memory acquisition and consolidation as well as impaired fear memory extinction (Milad et al. 2006, Quirk et al. 2006, Rauch et al. 2006), performing fear conditioning experiments with the aim of applying it to an animal model of PTSD, and relating these behavioural responses to a defined neural mechanism, will assist both in the elucidation of the underlying pathology of the disease, as well as the development of more effective treatment.

1.2 PROJECT AIMS

This project set about to re-examine the diverse and complex role of noradrenergic receptors in the conditioned fear response. However, while earlier studies have studied the role of noradrenergic receptors in fear conditioning using various models of conditioned fear as well as using various species of animal, this project considered various receptors of the noradrenergic system within the confines of a single study using a single fear conditioning model, and using a more sensitive animal.

The aims of this study were the following:

- To set up and validate a passive avoidance fear conditioning paradigm in rats for application in PTSD-related studies in our laboratory.
- To ascertain the role of various noradrenergic receptors in an avoidance paradigm of fear conditioning in rats.

The first objective was to validate the Gemini™ Avoidance System for use in our laboratory with regards to passive conditioned fear avoidance. Since passive fear conditioning is a central feature of PTSD, this aspect of the study would confer valuable face validity to the model under our conditions of study. Then, in order to facilitate the predictive (response to anti-adrenergic agent) and construct (adrenergic role) validity of the model with respect to the neurobiology of learning and fear, passive conditioned fear avoidance in the Gemini™ Avoidance System and its response to a centrally and a non-centrally active β-adrenergic blocking agent was assessed. The latter study would confirm the importance of a central mechanism of action for β-adrenoceptor blockade in the possible application of these drugs in preventing the development of PTSD posttrauma.

Once the model had been validated with respect to its response to a centrally and non-centrally active β-adrenergic blocking agent, the second objective was to use a non-pathological model to investigate the role of α_1-, α_2-, β_1-, and β_2-

receptors in a classical conditioned fear avoidance paradigm. This was done in order to investigate how selective pharmacological modulation of these receptors may modify the fear response, and whether any of these receptor systems may exert opposing effects on passive fear conditioning. To do this, I used various centrally active adrenergic agents, including the α_1-receptor antagonist, prazosin, the α_2-receptor agonist, guanfacine, the α_2-receptor antagonist, yohimbine, the β_1-receptor antagonist, betaxolol and the β_2-receptor antagonist ICI 118551.

Ultimately, knowledge obtained from this study can be used and applied to future exploratory studies in a translational animal model of PTSD which would investigate whether the tested agents described above may exert similar effects on conditioned fear responses in a PTSD-like paradigm. An example of such a translational model is single prolonged stress (SPS)(Liberzon et al. 1997, Liberzon et al. 1999a, Yamamoto et al. 2009) or time dependent sensitisation (TDS) (Harvey et al. 2003, Harvey et al. 2006). This work will shed light on the complex neurobiology of the fear response, and will have particular importance for our understanding of the neurobiology and progression to PTSD.

1.3 PROJECT LAYOUT

The project involved acute treatment with selective anti-adrenergic drugs in a 2 day, two-compartment, light-dark passive avoidance paradigm, measuring latency to avoid a foot shock. In all cases, a 3-tiered dose response analysis was performed in order to acquire reliable and robust behavioural data in response to drug treatment. In addition, locomotor activity of the animals was assessed in the pilot study on both days of testing. This was done in order to consider the possible effects of altered locomotor activity as a counfounding variable in the fear conditioning response.

Since Wistar rats have been described as showing more robust conditioned and unconditioned responses to stress (Staples, McGregor 2006), male Wistar rats (weighing 180-230g) were used in order to improve the outcome and

validity of this study. Importantly, studies in both animals (Cohen et al. 2004) and humans (Yehuda 2009) show that only 15-25% of trauma exposed individuals go on to develop PTSD, indicating that certain individuals are more sensitive to stress than others. In view of this, we employed behavioural cut-off criteria in accordance with the concept of "setting apart the affected" to select the more stress-responsive animals (Angelucci et al. 1999, Browman et al. 2005, Cohen et al. 2004, Cohen et al. 2005). In this way, animals were sifted to obtain a maximal response from 10 animals per treatment group.

To address the above-mentioned aims, appropriate noradrenergic drugs were tested using a three-tiered dose response design as follows:

Pilot study: Validation of the passive avoidance protocol using the GEMINI™ Avoidance System.

Validation of the passive avoidance paradigm and investigation of a central vs. peripheral mode of action was done using:

1. The centrally active non-selective β-antagonist, propranolol, at doses of 5, 10 and 20 mg/kg
2. The non-centrally active β-antagonist, nadolol, at doses of 2, 10 and 20 mg/kg

Main study: Investigation into the role of selective noradrenergic receptors in contextual passive avoidance fear conditioning.

Selective β-adrenergic responses were investigated using:

1. The β_1-selective antagonist betaxolol, at doses of 1, 5 and 10 mg/kg
2. The β_2-selective antagonist ICI 118551 at doses of 0.4, 1 and 4 mg/kg

Selective α-adrenergic responses were investigated using:

1. The α_1-antagonist, prazosin, at doses of 0.1, 1 and 5 mg/kg
2. The α_2-antagonist, yohimbine, at doses of 1, 5 and 10 mg/kg
3. The α_2-agonist, guanfacine, at doses of 0.1, 0.3 and 1 mg/kg

Saline was used as control and the drugs were injected subcutaneously on day 1 immediately following fear conditioning.
n=10 for each treatment cohort.

Passive avoidance fear conditioning was conducted in the Gemini™ Avoidance System (San Diego Instruments). On day one animals were subjected to a training trial, in which they must learn to fear the dark compartment of the apparatus (a compartment they would otherwise normally prefer) due to the administration of a foot shock in that compartment. The cross-over latency or time (in seconds) was then recorded. Immediately following this training trial, the appropriate drugs were administered subcutaneously and the animals returned to their home cages. Twenty-four hours later, the procedure was repeated in the retention trial and the cross-over latency recorded again. No drugs were administered on the second day of fear conditioning.

In order to exclude possible confounding effects of differences in inherent locomotor activity of the animals, which could possibly affect the fear conditioning response, general locomotor activity was assessed in the pilot study using the Digiscan™ Animal Activity Monitor (Omnitech Electronics, Columbus, OH). To do this, a five minute locomotor assessment trial was conducted five minutes prior to both the training and retention trials in the fear conditioning paradigm. If altered locomotor activity proved not to be a confounder under our conditions of study, this test would not be undertaken in the main study.

The sequence of events on both day one and day two was thus as follows:

Pilot study:

1. 5 minute locomotor assessment trial in the Digiscan Animal Activity Monitor (day 1)
2. Passive avoidance training in the Gemini™ Avoidance System (day 1)
3. Acute subcutaneous drug administration with either saline or one of the noradrenergic active drugs, only on day one of fear conditioning (day 1)
4. 5 minute locomotor assessment trial in the Digiscan Animal Activity Monitor (day 2)
5. Recall of fear conditioned response in the Gemini™ Avoidance System (day 2)

Main study:

1. Passive avoidance training in the Gemini™ Avoidance System (day 1)
2. Acute subcutaneous drug administration with either saline or one of the noradrenergic active drugs, only on day one of fear conditioning.
3. Recall of fear conditioned response in the Gemini™ Avoidance System (day 2)

1.4 GENERAL POINTS

This dissertation has been prepared in the article format as approved for submission by the North-West University. The article was written in accordance with the instructions to the authors in the house style of the selected journal, provided in Addendum B of this dissertation. The article format of this dissertation consists of an introductory chapter that briefly summarizes the background and motivation for the study, as well as the study aims and project layout. The second chapter gives an overview of the literature on the research subject, while the manuscript, prepared for submission to a peer-reviewed

journal, will be presented in the third chapter. In the final chapter, a discussion of both the pilot and main studies' results are presented, as well as conclusions and recommendations for further studies. The pilot study is presented as Addendum A at the back of this dissertation.

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CHAPTER 2 Literature Review

2.1 POSTTRAUMATIC STRESS DISORDER

Posttraumatic stress disorder (PTSD) is a chronic, debilitating and often very severe psychiatric disorder that can develop following any traumatic or life-threatening event in which extreme horror or intense fear were experienced. It was introduced as a diagnosis in 1980 and is categorised as an anxiety disorder in the Diagnostic and Statistical Manual of Mental Disorders (4th Edition Text Revision) (American Psychiatric Association 2000). The prevalence rate of this anxiety disorder varies according to the trauma exposure of a particular population, with a frequency rate of approximately 7% found in the general population in the USA (Kessler et al. 2005) but with a prevalence as high as 24% in certain subpopulations, such as combat veterans (Milliken et al. 2007). Results from numerous studies indicate that trauma exposure rates among adolescents vary between 82% and 100%, with corresponding PTSD rates ranging from 6% to 22% (Fincham et al. 2009, Seedat et al. 2004a, Ward et al. 2001). Approximately 15-25% of trauma exposed individuals go on to develop PTSD (Yehuda 2009), depending on the nature and severity of the trauma (Afifi et al. 2010, Stein et al. 2002, True et al. 1993).

2.1.1 AETIOLOGY

PTSD can occur in the aftermath of any traumatising or life-threatening event in which intense fear, horror or bodily harm was incurred. Numerous risk factors for developing PTSD have been postulated, including the nature, severity and duration of the traumatic event, childhood trauma, genetic factors, inadequate social support and pre-existing behavioural pathology (Afifi et al. 2010, Stein et al. 2002, True et al. 1993). However, taking into perspective neurobiological and neuroanatomical deficits observed in PTSD, it still remains to be

ascertained whether these deficits are a result of PTSD, as opposed to being a risk factor for PTSD. In line with this train of thought, shrinkage of the hippocampus has been found in patients with PTSD and has been proposed to be a consequence of the maladaptive stress response (Bremner 2001, Sapolsky 2000). On the other hand, Gilbertson and colleagues' study on twin combat veterans suggested that a smaller hippocampal volume may rather be a premorbid risk factor for the development of PTSD than a consequence thereof (Gilbertson et al. 2002). Similarly, decreased amygdalar and anterior cingulate cortex volumes have been shown in PTSD patients, and the question whether these changes represent aetiological factors or non-specific predispositional factors remains a point of discussion (Karl et al. 2006). Alteration in glucocorticoid secretion also suggests a dysfunctional HPA axis as possible causative pathophysiology in PTSD, although again the precise contribution of this system to the pathology of PTSD is still under scrutiny (Charney et al. 1993, Yehuda 2001).

At the most basic level, failure to recover and adapt from responses to stress as well as dysfunctional extinction of fearful memories is regarded as the main reason for the progression of acute traumatic stress syndrome to full blown PTSD.

2.1.2 SYMPTOMATOLOGY, COMORBIDITIES AND IMPACT ON QUALITY OF LIFE

PTSD is considered a maladaptive fear response to a traumatic stressor, and is characterised by three symptom clusters, namely re-experiencing, hyperarousal and avoidance/numbing behaviour, with numbing behaviour recently being suggested as a fourth distinct symptom cluster (Olff et al. 2009).

Hypermnesic recall of the event results in re-experiencing in the form of frequent flashbacks, nightmares and intrusive thoughts, while memory for peritraumatic events is often disturbed. These intrusions are often very distressing to the patient, and can be detrimental to the patient's social functioning, explaining why this symptom cluster is often the reason patients seek psychological help (Steil, Ehlers 2000).

Hyperarousal refers to symptoms such as difficulty sleeping, exaggerated startle response, irritability and outbursts of anger, concentration problems and hypervigilance. This component of the symptom triad can be attributed to increased anxiety-like behaviour resulting from unresolved fear responses to the original trauma (American Psychiatric Association 2000) .

Avoidance behaviour can include symptoms such as actively or passively avoiding places, people and events reminiscent of the trauma and forgetting specific details of the trauma. Numbing behaviour (included as part of the avoidance behaviour cluster in the DSM-IV-TR, 2000) may be expressed in feelings of detachment, inability to express loving feelings toward others or feeling emotionally blunted, as well as having a shortened sense of the future (American Psychiatric Association 2000). This greatly incapacitates the patient's relationships as well as their ability to work and function normally in a social environment.

Apart from the symptom triad, PTSD is also characterised by cognitive impairments, including impaired attention and working memory function (Weber et al. 2005, Vasterling et al. 1998), impaired explicit memory (such as short-term memory loss) (Rauch et al. 2006, Shin et al. 2004) and impediments in new learning and memory acquisition (Golier et al. 2002).

These symptoms greatly impact the general well-being and social activities of the patient (Yehuda 2002). Indeed, Kessler found that patients suffering from PTSD have a six times higher probability of committing suicide, an increased risk of dropping out of school and are more prone to experience marital instability, and that overall work impairment is comparable to that found in patients who suffer from major depressive disorder (Kessler 2000). PTSD presents with many comorbidities while the lifetime comorbidity between PTSD and major depression has been found to be as high as 50% (Kessler et al. 1995). Furthermore, PTSD patients are more likely to fall prey to substance abuse and smoking (Karney et al. 2008) and reports indicate a high incidence of divorce and domestic violence under sufferers of PTSD (Byrne, Riggs 1996, Jordan et al. 1992). Finally, comorbid psychiatric diagnoses of depression, anxiety or substance abuse has been shown to be as high as 88% in men and 79% in women with PTSD (Kessler et al. 1995).

Woods and colleagues showed a definite correlation between problems with physical health and symptoms of PTSD (Woods, Wineman 2004). It is therefore evident that the patient suffering from chronic PTSD has an increased risk of suffering from various other psychological, social and physical problems, impoverishing their sense of meaning and pleasure and severely affecting their quality of life, with socio-economic implications that affect both the patient and his family.

2.1.3 DIAGNOSTIC CRITERIA

As mentioned earlier, not all patients who are victims of traumatic events go on to develop the syndrome of PTSD. Symptoms of PTSD become evident when the presentation of acute traumatic stress symptoms does not dissipate, and is a direct result of maladaptive responses to stress and traumatic memories. The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) has described the diagnostic criteria for PTSD. These criteria have been reproduced below from the 4th Edition Text Revision (American Psychiatric Association 2000):

Criterion A: Stressor

The person has been exposed to a traumatic event in which both of the following have been present:

1. The person has experienced, witnessed, or been confronted with an event or events that involve actual or threatened death or serious injury, or a threat to the physical integrity of oneself or others.

2. The person's response involved intense fear, helplessness, or horror. Note: in children, it may be expressed instead by disorganized or agitated behavior.

Criterion B: Intrusive Recollection

The traumatic event is persistently re-experienced in at least one of the following ways:

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

2. Recurrent distressing dreams of the event. Note: in children, there may be frightening dreams without recognizable content.

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

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

5. Physiologic reactivity upon exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event.

Criterion C: Avoidance/Numbing

Persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness (not present before the trauma), as indicated by at least three of the following:

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

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

3. Inability to recall an important aspect of the trauma

4. Markedly diminished interest or participation in significant activities

5. Feeling of detachment or estrangement from others

6. Restricted range of affect (e.g., unable to have loving feelings)

7. Sense of foreshortened future (e.g., does not expect to have a career, marriage, children, or a normal life span)

Criterion D: Hyper-arousal

Persistent symptoms of increasing arousal (not present before the trauma), indicated by at least two of the following:

1. Difficulty falling or staying asleep

2. Irritability or outbursts of anger

3. Difficulty concentrating

4. Hyper-vigilance

5. Exaggerated startle response

Criterion E: Duration

Duration of the disturbance (symptoms in B, C, and D) is more than one month.

Criterion F: Functional Significance

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

In order to make a diagnosis of PTSD, the presence of a number of symptoms from all three symptom clusters (as described in section 2.1.2 and above) is required as follows: one re-experiencing symptom, three avoidance symptoms and two hyperarousal symptoms. In addition, these symptoms should be present for at least one month, and should cause significant disruption to the patient's personal, social or occupational functioning. Symptoms that are present for less than 3 months are considered acute PTSD, and chronic PTSD is diagnosed if these symptoms last for longer than 3 months (Feliciano 2009).

2.1.4 THE STRESS RESPONSE

The stress response is the result of an orchestrated response mediated by the amygdala, the PFC, hippocampus and the HPA axis (Dedovic et al. 2009). Figure 1 on page 27 gives a graphical presentation of the neurobiology of the stress response. Upon presentation of a threat, the amygdala is activated via the thalamo-amygdala pathway (LeDoux 1992), which is a sub-cortical pathway allowing for a fast impression of the threat without recruiting cognitive processes, causing an immediate emotional (fearful) response before the perceived threat has been fully evaluated. The HPA axis is activated, which triggers the production and release of glucocorticoids and adrenaline from the adrenal cortices (Jacobson, Sapolsky 1991). The HPA system also triggers the release of the catecholamines dopamine and noradrenaline. These stress hormones are important in facilitating and consolidating strong emotional memories in the hippocampus. The catecholamines suppress activity in the prefrontal cortex, which is responsible for short-term memory, inhibition, concentration and rational thought. This dampens inhibitory behaviour, which

results in quick, amplified responses to the fearful situation, and inability to handle social and intellectual demands at the time. The release of the protein, neuropeptide S, modulates the stress response by increasing alertness and the sense of anxiety, which directly urges the fight or flight response. The hippocampus is then stimulated to store emotionally loaded experiences in long-term memory, so as to be able to recall this potential threat in the future and react to it appropriately (Herman et al. 2005).

Under normal conditions of stress, the above cascade of events manifests in fearful reactions. Soon after escaping from the threatening situation, normal function resumes. However, in PTSD the brain's adaptation to the environment doesn't resume usual function, resulting in overly responsive neural fear responses and increased fear memory acquisition and consolidation (Bremner 2002). The action of the amygdala is strengthened (Liberzon et al. 1999b), while the PFC and hippocampus are impaired (Bodnoff et al. 1995, Luine et al. 1994), resulting in impaired encoding of new memories and weaker extinction of the fear memory.

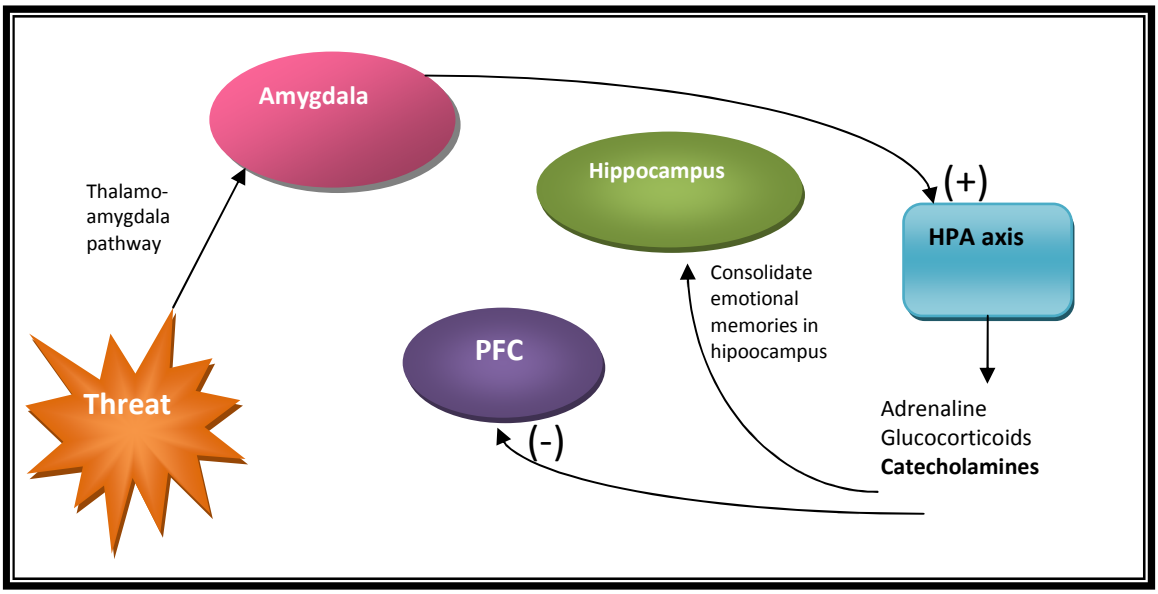

Figure 1: Graphical presentation of the stress response. Upon presentation of a threat, the stimulus is processed along the quick, subcortical thalamo-amygdala pathway to the amygdala, which stimulates the HPA axis to secrete stress hormones. Catecholamines secreted by the HPA axis inhibits the inhibitory function of the PFC and facilitates consolidation of emotional memories in the hippocampus. See section 2.1.4 for a more detailed description.

2.1.5 The Neuroanatomy of PTSD

Functional neuroimaging studies and basic research has helped to identify three brain regions possibly involved in the pathology of PTSD, viz. the hippocampus, amygdala and medial prefrontal cortex (mPFC), and functional relationships between these three brain areas have also been identified (Bremner et al. 2005, Shin et al. 2006). Moreover, a functional relationship between the HPA axis and the aforementioned brain areas have also been established (Dedovic et al. 2009, Herman et al. 2005).

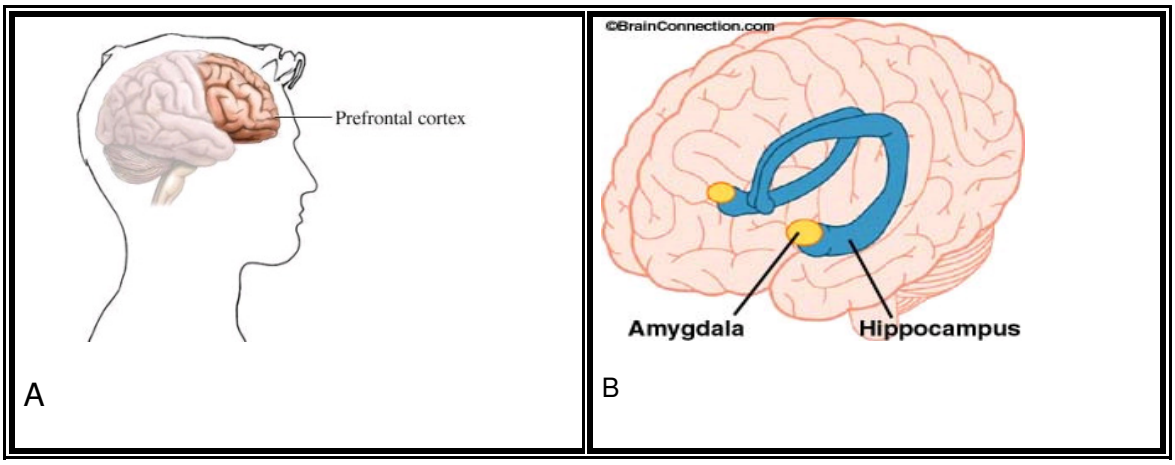

Figure 2: A) Location of the PFC and B) the amygdala and hippocampus. Adapted from: www.brainconnections.com

2.1.5.1 The Hippocampus

The hippocampus is a paired structure belonging to the limbic system and is located inside the medial temporal lobe, just beneath the surface of the cortex (see Fig. 2B).

The hippocampus plays a vital role in consolidation of short-term memory into long-term memory. It is also important in the regulation of the stress response (Jacobson, Sapolsky 1991) and in the function of working/declarative memory (Bremner 1999, Elzinga, Bremner 2002). Due to the high density of glucocorticoid receptors in the hippocampus (McEwen, Wallach 1973, Morimoto et al. 1996), as well as the neurotoxic effects of continual high levels of glucocorticoids in this brain structure (Uno et al. 1994), the hippocampus is very sensitive to the deleterious effects of stress (Joëls 2008). Thus for example, Llorente and colleagues showed neuronal alterations and degradation in the hippocampus of post-natal rats subjected to maternal separation stress (Llorente et al. 2009). Accordingly, many studies have shown significantly smaller hippocampal volumes in PTSD patients (Bremner et al. 1995, Bremner et al. 1997, Smith 2005, Stein et al. 1997a), while various meta-analyses of structural brain abnormalities in PTSD support these findings (Karl et al. 2006, Kitayama et al. 2005, Lanius et al. 2006). This shrinkage of the hippocampus has been suggested to be responsible for the impairment in new

learning in patients with PTSD, the inability to deal with intrusive memories (Bodnoff et al. 1995, Luine et al. 1994) as well as deficits in regulating the stress response. In support of this hypothesis, Pruessner and colleagues noted an inverse correlation between hippocampal volume and the cortisol response to a neuroimaging stress task (Pruessner et al. 2005). In an earlier study in rats, removal of the hippocampi produced an animal with an increased sensitivity to mild stress (Kant et al. 1984). The hippocampus may also play a role in the interpretation of the intensity of a stressor (Herman et al. 2005), leading to the conclusion that reduced hippocampal volume in PTSD patients may be the cause of exaggerated startle responses and maladaptive stress responses seen in these patients.

2.1.5.2 The Amygdala

The amygdala (see Fig. 2B) plays a prominent role in the processing and memory of emotional events and forms a critical part of the limbic system. The amygdaloid bodies are almond-shaped groups of nuclei located in the medial temporal lobe. The amygdala is involved in the formation and retrieval of emotional and aversive memories (Shin et al. 2006) and is important in the extinction of learned fear (Meyers, Davis 2007). It is also thought to play a vital role in the modulation of long-term memory consolidation in other brain areas, possibly via LTP, and amygdalar lesions have been associated with impairment in fear conditioning (Killcross et al. 1997, Killcross 2000). Furthermore, the amygdala is believed to be the activator of the HPA axis (Herman et al. 2005), which is one of the key catalysts of the stress response. Research has provided evidence for amygdala hyperresponsivity in PTSD (Liberzon et al. 1999b), as well as the hypothesis that amygdalar glucocorticoid receptors potentiate HPA responses (Dallman et al. 2006). This correlates with the conceptualization of a hyperresponsive HPA axis in PTSD. When presented with trauma-related images, positron emission tomogrophy (PET) demonstrated that the regional cerebral blood flow (cRBF) in the amygdala in patients with PTSD is increased compared to controls (Shin et al. 2004), which in turn was negatively correlated with cRBF in the medial frontal gyrus. Functional magnetic resonance imaging (MRI) studies also showed that PTSD patients exhibit exaggerated amygdalar

responses to fearful versus happy facial expressions when compared to controls (Shin et al. 2005). Diminished mPFC activity was also noted in this study, providing further evidence for a reciprocal negative correlation between amygdala and prefrontal cortex activity in PTSD. Structural abnormalities of the amygdala in patients with PTSD have also been noted, with left amygdalar volumes being significantly smaller in patients with PTSD (Karl et al. 2006, Rogers et al. 2009, Shin et al. 2004). Changes in neuronal plasticity in fear-related subdivisions of the amygdala also appear to be associated with trauma-induced social deficits in PTSD (Mikics et al. 2008). The status quo is therefore that the amygdala shows excessive responsiveness to stress in PTSD, which is correlated to diminished inhibitory activity in the PFC.

2.1.5.3 The Prefrontal Cortex (PFC)

The PFC (see Fig. 2A) plays an important role in working memory function (Ramos, Arnsten 2007) and is also responsible for inhibiting the response of the amygdala to stress (Shin et al. 2004). PTSD has been associated with an overactive amygdala and impaired PFC function (Bremner 2002). On the other hand, the medial prefrontal cortex (mPFC) has a modulating role in emotional processes such as conditioned fear extinction (see section 2.3 for detail on fear conditioning and section 2.2.3 for detail on fear extinction) (Quirk, Beer 2006) and exerts indirect inhibitory control over HPA axis responses (Radley et al. 2009). Indeed, PTSD has been associated with an exaggerated HPA axis negative feedback response (Kohda et al. 2007, Liberzon et al. 1999a, Liberzon et al. 1999b), which provides further evidence of impaired PFC functioning. In line with this, Pruessner and colleagues found that individuals who exhibit a significant stress response showed decreased activity in this brain area (Pruessner et al. 2008). It therefore seems that PTSD is characterised by alterations in PFC neural activity. A meta-analysis of structural brain abnormalities in PTSD also concludes that PTSD is associated with significantly smaller prefrontal lobe volumes (Karl et al. 2006, Shin et al. 2004).
Therefore, from an overview of the above literature, it seems that PTSD is associated with decreased volumes in the hippocampus, amygdala and PFC, and that the function of the hippocampus and PFC is impaired in PTSD, while

the activity of the amygdala is increased. These structural and functional abnormalities are depicted in Figure 3.

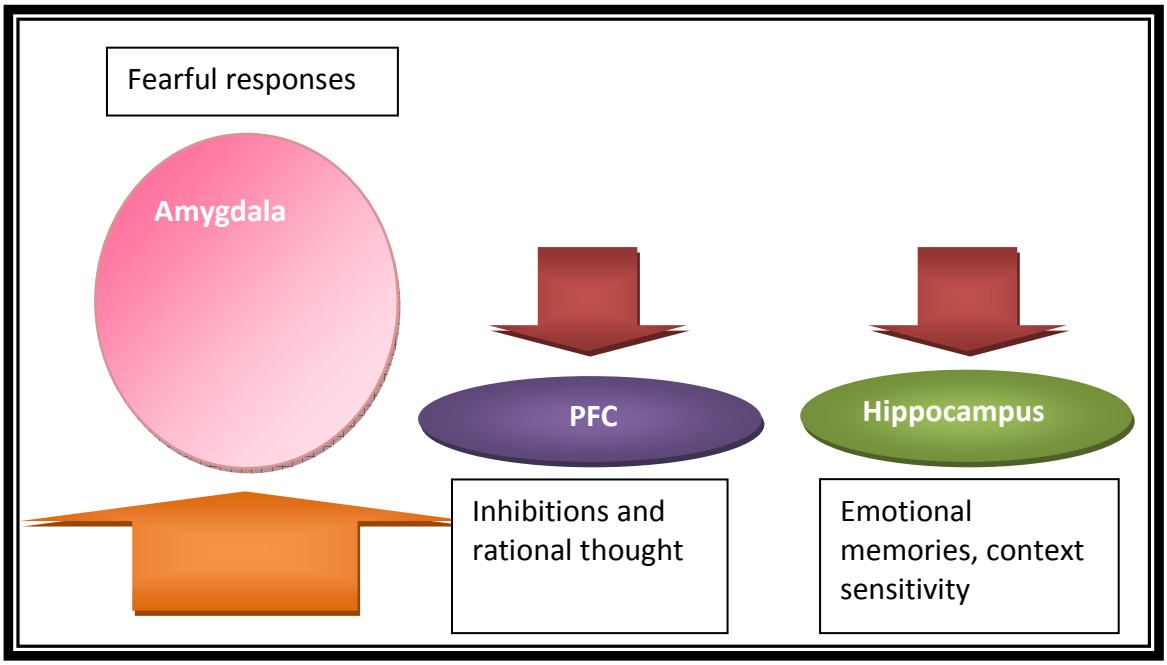

Figure 3: Graphical presentation of structural brain abnormalities in PTSD. The amygdala is hyperresponsive to stress, while the function of the hippocampus and PFC is impaired, resulting in exaggerated fearful responses, diminished rational and inhibitory behaviour and distorted emotional memories lacking context sensitivity.

2.1.6 The Neurobiology of PTSD

Dysfunctional activity of multiple neurobiologic pathways have been implicated in the pathology underlying PTSD symptomotology, including the noradrenergic, serotonergic, dopaminergic and glutamatergic systems, as well as the HPA axis. These systems are also reciprocally interlinked, with dysfunction of one system affecting the function of the other, thereby significantly complicating the pathology of the disorder and demanding investigation to delineate the roles of each system in the neuropathology of PTSD.

2.1.6.1 Noradrenergic involvement

The noradrenergic system in conjunction with cortisol, plays a vital role in the stress response and in the learning and memory of fear. Noradrenergic

neurons project from the locus coeruleus (LC) in the brainstem to several brain areas implicated in the stress response and in memory, including the PFC, hippocampus, amgydala and thalamus. The LC has the vital task of organising the affective, cognitive and motor responses to acute stress, and activation of the LC leads to secretion of noradrenaline (Olpe et al. 1985). Many of the symptoms exhibited by patients with PTSD are suggestive of a hyperadrenergic state, such as hyperarousal, hypervigilance, sleeplessness and an exaggerated startle response. Indeed, increased urinary levels of noradrenaline have been reported in combat veterans with PTSD compared to those without PTSD (Yehuda et al. 1992), as well as in women with PTSD and a history of sexual abuse, as compared to women without these psychopathologies (Lemieux, Coe 1995). These results have been mirrored in other populations with PTSD studying 24-hour catecholamine excretion in urine (De Bellis et al. 1994, Kosten et al. 1987). Furthermore, elevated levels of NA were observed in the cerebrospinal fluid of male combat veterans with chronic PTSD that also corresponded to symptom severity (Geracioti et al. 2001). Further evidence for a hyperadrenergic state in PTSD comes from studies showing hypersensitivity of adrenergic receptors in patients with chronic PTSD (Morgan et al. 1993a, Southwick et al. 1993), while Perry and colleagues showed a reduction in platelet α_2-adrenergic receptors in PTSD patients, which will further amplify the response to already elevated NA levels (Perry et al. 1987).

Normally NA has a lower affinity for α_1-receptors than for α_2-receptors. In the PFC and during a state of moderate NA release, NA will preferentially bind the high-affinity α_2-receptors which will in effect improve PFC function (Ramos, Arnsten 2007). Where higher NA levels are present, as in the case following a severe emotional trauma, low affinity α_1-receptors are engaged which will impair PFC function. Since the PFC has an important inhibitory role over the amygdala, impaired PFC function will result in impaired inhibitory tone on the amygdala which in turn will increase the emotional response to stress and so enhance fear conditioning and give rise to hyperarousal typically observed in PTSD.

Noradrenergic β-receptors have been shown to be essential in emotional learning and memory of fear (Pitman et al. 2002, Roozendaal et al. 2004). Non-selective blockade of β-receptors have repeatedly produced attenuated fear acquisition and fear conditioning in humans and animals (Cahill et al. 1994, Pitman et al. 2002, Roozendaal et al. 1997). However, where non-selective β-receptor antagonism with propranolol has shown no effect on PFC function in monkeys, selective antagonism of the β_1-receptor seemed to enhance PFC function (Ramos et al. 2005). This might suggest separate roles for the β_1- and β_2-receptors in PTSD, especially in the light of propranolol's modest clinical efficacy in preventing the onset of PTSD (Cohen et al. 2011, McGhee et al. 2009, Sharp et al. 2010, Stein et al. 2007). Since the PFC exerts inhibitory control over the hippocampus, amgydala (Shin et al. 2004) and the HPA axis (which exhibits exaggerated responses in PTSD (Radley et al. 2009)) selectively targeting the β_1-receptor in PTSD could result in greater control over traumatic memories and improved context sensitivity. The notion of distinct roles for the β_1- and β_2-receptors is supported by Schutsky et al (2011), who showed that memory retrieval is dependent on β_2- but not β_1-adrenergic receptors. The authors conclude that the β_2-receptor can be a critical effector of acute stress, and that β_1- and β_2-receptors can have distinct roles in central nervous system (CNS) signaling and cognition (Schutsky et al. 2011). Indeed, in studies on stress-induced cocaine re-instatement in rats, selective β_2-antagonism, but not selective β_1-antagonism, blocked forced swim stress-induced cocaine reinstatement (Vranjkovic et al. 2011). This might suggest that β_2-antagonists exert a more potent effect on inhibiting stressful responses. Indeed, in a study in combat veterans with PTSD, the authors reported increased neutrophil β_2-receptor density and enhanced β_2-receptors G-protein coupling (Gurguis et al. 1999).

The noradrenergic system also plays a prominent role in activating the HPA axis and the role of glucocorticoids in the stress response (Roozendaal et al. 2006). A reciprocal modulation across noradrenergic and glucocorticoid systems has been demonstrated, where an increase in noradrenaline drives an

increase in CRF release and vice versa (Dunn et al. 2004, Gresack, Risbrough 2010).

2.1.6.2 The HPA axis and the role of glucocorticoids

Under normal physiological conditions, the hypothalamic-pituitary-adrenal axis responds to stress by increasing the secretion of corticotrophin releasing factor (CRF) from the hypothalamus, which stimulates the secretion of adrenocorticotrophin releasing hormone (ACTH) from the pituitary. ACTH in turn stimulates the release of cortisol from the adrenal cortices. Negative feedback control of cortisol on the pituitary gland and hypothalamus then terminates the bio-behavioural stress response and is critical in curbing the deleterious effects of excessive glucocorticoids on the brain and other organs. Although cortisol levels are increased immediately following the trauma, PTSD is associated with unexpectedly low cortisol levels (Parker et al. 2003). Indeed, studies in patients with PTSD have indicated cortisol 'super-suppression' (Stein et al. 1997b) and an increased density of glucocorticoid receptors on peripheral lymphocytes, suggesting that hypocortisolaemia in PTSD is due to an exaggerated HPA axis negative feedback response (Liberzon et al. 1999a) as well as CRF hypersecretion (Baker et al. 1999). Hypersecretion of CRF results in a blunted ACTH response to CRF, in turn resulting in a decrease in the release of cortisol from the adrenal cortices. Since glucocorticoid release is responsible for inhibiting noradrenaline secretion from the sympathetic nerve terminals (Pacak et al. 1995), lower cortisol levels may prolong the availability of noradrenaline in adrenergic synapses resulting in failure in shutting off the stress response (Yehuda 1997).

Cortisol plays a critical role in the interaction between the hippocampus and amygdala during the encoding of emotional memory (Wolf 2008), particularly through its activation of the glutamatergic system (Takahashi et al. 2002). Thus, lower cortisol levels in combination with enhanced noradrenergic tone might be a cause for the memory intrusions, flashbacks and nightmares commonly found in PTSD. Indeed, PTSD patients show decreased basal corticosteroid levels (Yehuda et al. 1991). While fear memory will in this way be facilitated,

prolonged glutamatergic activity will also lead to excitotoxic damage to neurons of the hippocampus, leading to neuronal atrophy and attrition of explicit memory performance and other cognitive deficits. During acute stress, elevated glucocorticoid levels work together with noradrenergic activation in the amygdala to enhance the encoding and consolidation of emotional memories (Roozendaal et al. 2006). The hippocampus and PFC usually inhibit HPA axis secretion, while the amygdala is implicated in activating the secretion of glucocorticoids (Feldman et al. 1995, Jacobson, Sapolsky 1991). The mPFC is rendered ineffective during acute stress (Hurlemann 2008), and since this brain region exerts an inhibitory action over the noradrenergic neurons of the locus coeruleus and the HPA axis, stress will sensitize amygdala activation by glucocorticoids and noradrenaline leading to a strengthening of fear memory consolidation (Hurlemann 2008). The hippocampus is implicated in the inhibition of the HPA axis (Herman, Cullinan 1997, Jacobson, Sapolsky 1991), and with evidence of impaired hippocampal function in PTSD (see section 2.1.5.1), this then provides further support for dysfunctional HPA axis regulation in PTSD.

Although PTSD is associated with hypocortisoleamia, initial over-exposure to glucocorticoids in the immediate aftermath of the trauma could account for structural abnormalities in the brain regions discussed under section 2.1.5 (Shalev et al. 2008, Simeon et al. 2007). Excessive amounts of glucocorticoids have been shown to have deleterious effects on the central nervous system and cognition (McEwen, Sapolsky 1995, Sapolsky 1999, Uno et al. 1994). In animal studies, prolonged exposure to glucocorticoids or stress causes atrophy of dendritic branches in pyramidal neurons in the rat hippocampus (Watanabe et al. 1992, Woolley et al. 1990), potentiates neural damage induced by kainic acid (Stein-Behrens et al. 1992) and potentiates the release of excitatory amino acids in the hippocampus (Stein-Behrens et al. 1994). Extended periods of exposure to glucocorticoids can even result in hippocampal neuron loss in the rat (Sapolsky et al. 1985). In an animal model of posttraumatic stress disorder, Kozlovsky et al. (2009) suggest that persistently increased levels of glucocorticoids, which is associated with decreased levels of the neuro-

protective agent brain-derived neurotrophic factor (BDNF), might mediate changes in neural plasticity and synaptic function underlying chronic stress-induced psychopathological processes.

2.1.6.3 The role of dopamine

Dopamine is also a catecholamine that has been widely studied in the paradigms of fear learning and memory. The amygdala is richly innervated by dopaminergic neurons (Oades, Halliday 1987), while stress evokes a significant increase in extracellular dopamine in the amygdala (Oshibuchi et al. 2009). Studies in animals have shown that over-activation of dopaminergic transmission may exacerbate conditioned fear (Bissiere et al. 2003, Debiec, LeDoux 2006, Inoue et al. 2000). Furthermore, quinpirole, a full D_2-receptor agonist, blocks the acquisition of second-order fear conditioning while sulpiride, a selective D_2-receptor antagonist, facilitates fear memory extinction (Nader, LeDoux 1999). Thus these data would suggest that dopamine D_2-receptors are necessary for fear learning (Fadok et al. 2009) while dopamine is necessary for cue-dependent fear conditioning. Consequently, D_2-receptor stimulation would strengthen fear conditioning, whereas a D_2-antagonist should inhibit fear conditioning. Although dopamine's role in fear conditioning has been studied, the specific role of dopamine in a model of PTSD has not been fully elucidated. In a recent study using a time dependent sensitization model, Harvey and colleagues found a pronounced elevation of DA in the frontal cortex 1 hour after the initial trauma, but a significant decrease in DA levels was observed 7 days after re-stress (Harvey et al., 2006). This suggests that the initial trauma and a subsequent re-stress (related to re-experience in PTSD) evoke qualitatively different effects on dopamine that may have relevance as to how this transmitter is viewed with regards to treatment and intervention strategies. Decreased dopaminergic activity in the frontal cortex has been suggested to compromise the ability to develop effective coping strategies in dealing with trauma (see Harvey et al., 2006 for discussion) thereby contributing to hyper-vigilance and greater susceptibility to trauma-related contextual stimuli. Stam (2007) suggests that due to dopamine's involvement in memory encoding, it

may have a role in the subsequent failure of extinction or unsuccessful coping in patients afflicted with PTSD (Stam 2007a, Stam 2007b). Dopamine D_1 receptors may also have a role to play, since high levels of dopamine, acting at dopamine D_1-receptors, lead to impairment of PFC function (reviewed in Robbins and Arnsten 2009).

2.1.6.4 The role of serotonin

The role of serotonin in the pathophysiology of PTSD is less defined than that of the noradrenergic system, and the effective use of pro-serotonergic agents in the treatment of certain PTSD symptoms has been the main basis of support for hypotheses for an underlying serotonergic dysfunction in the disorder. Clinical studies employing MRI have demonstrated that chronic administration of paroxetine (a SSRI) to patients with PTSD was associated with an increase in hippocampal volume, in addition to effectively treating certain symptoms of PTSD (Vermetten et al. 2003). Furthermore, in a study consisting of 8 weeks of citalopram administration to PTSD patients, single photon emission computed tomogrophy (SPECT) showed a correlation between symptomatic improvement and activation of the medial prefrontal cortex, suggesting that serotonergic agents improve PTSD symptoms by correcting mPFC inhibitory action on the amygdala (Seedat et al. 2004b). A reciprocal connection between the serotonergic system and noradrenergic system also exists, with serotonin serving to dampen the firing of noradrenergic neurons in the locus coeruleus (Szabo et al. 1999), which supports the notion of enhanced noradrenergic activity in PTSD. Furthermore, decreased serum concentrations of serotonin (Spivak et al. 1999) as well as a decreased density of platelet serotonin uptake sites in PTSD patients (Maes et al. 1999), support a hypothesis of decreased serotonergic activity in PTSD. Serotonin also affects the HPA axis and Jensen and colleagues found that long-term administration of SSRIs in rats appears to attenuate HPA axis responsiveness (Jensen et al. 1999). Therefore, empirical and pharmacological data suggest decreased serotonergic activity in PTSD. On the other hand, some pre-clinical work has suggested a dual role for serotonin, that excessive serotonergic activity may in fact also be detrimental under

certain conditions (Czéh et al. 2001, Harvey et al. 2004, Harvey 2011, Kalynchuk et al. 2001, Kawahara et al. 1993). This emphasises the fact that further work is needed to delineate the role of this transmitter in the neurobiology and treatment of stress related disorders such as PTSD.

2.1.6.5 The role of glutamate

Stress and glucocorticoids each in their own right increase the release of glutamate in the CNS where it plays a pivotal role in learning and memory processes (Riedel et al. 2003). Glutamate exerts a bimodal action on memory function, with too low levels negatively influencing memory formation and excessively high levels adversely affecting memory due to the excitotoxic nature of this amino acid (Lowy et al. 1995). Indeed, glutamate toxicity has been suggested to underlie hippocampal damage in PTSD (Stein-Behrens et al. 1994). Glutamate is involved in fear and stress responses (Davis, Myers 2002, Miyamoto et al. 2002, Walker et al. 2002) and in various processes underlying anxiety (Harvey, Shahid 2011, Kessler et al. 1995, Moghaddam 1993). The glutamate NMDA-receptor especially has been implicated in the process of fear extinction (Baker, Azorlosa 1996, Falls et al. 1992), with inhibition of this receptor blocking the acquisition of fear conditioning (Cammarota et al. 2004, Joca et al. 2007). Studies in an animal model of PTSD have not only observed changes in both NMDA-receptor binding as well as reduced gamma-amino butyric acid (GABA) levels in the rat hippocampus, but have also demonstrated the activation of nitric oxide synthase (NOS), a down-stream signaling molecule for the NMDA receptor (Harvey et al. 2004, Harvey et al. 2005). Of particular interest is that induced acoustic startle response in a rodent model of PTSD can be attenuated by topiramate, an anti-epileptic with glutamate-modulating properties (Khan, Liberzon 2004). Similarly, the NMDA-receptor antagonist ketamine produces dissociative-like symptoms in patients that is also commonly seen in PTSD, thus suggesting that a hyperglutamatergic state may be present in PTSD (Chambers et al. 1999, Krystal et al. 1994). These studies confirm that the encoding and consolidation of traumatic memories, at least in rodents, are dependent on the activation of glutamatergic-

dependent signaling (Joca et al. 2007). Thus, a hyperglutamatergic state might be an important neurobiological mechanism in PTSD.

Summarizing, hypernoradrenergic, hyperdopaminergic, hyperglutamatergic and hyposerotonergic activity is implicated in PTSD neuropathology, with reduced baseline cortisol levels and a hyperresponsive HPA negative feedback response are intricately connected to each neurotransmitter system. These alterations in multiple neurochemical pathways and their interconnectivity and reciprocal interaction is part of the complicating factors in PTSD neuropathology, necessitating further investigation into the clarification of the role of each subsystem.

2.1.7 CURRENT PHARMACOLOGICAL TREATMENT STRATEGIES

Current pharmacological treatment strategies for PTSD are focused primarily on preventative intervention posttrauma and on the treatment of chronic PTSD symptoms, and are divided into three different approaches: firstly, primary and secondary prevention of the neurodevelopment of PTSD, secondly treatment of non-cognitive symptoms of PTSD, and thirdly the treatment of cognitive symptoms of PTSD (Steckler, Risbrough 2011).

2.1.7.1 Prevention of the neurodevelopment of PTSD

This approach is complicated by concerns that only a small population of trauma exposed individuals go on to develop PTSD (Yehuda 2009). Furthermore, while preventative pharmacological approaches should aim to effectively counteract the neurodevelopment of PTSD symptomatology, normal function and psychological responses to traumatic events should be left undisturbed and cognitive and psychomotor function should not be impaired.

2.1.7.1.1 Primary pharmacological prevention

Primary pharmacological prevention focuses on enhancing the individual's ability to cope with stress, therefore to facilitate resilience to stress. This, of

course, would only be a viable option in cases where the individual will undoubtedly be subjected to stressors that may induce symptoms of PTSD, such as soldiers sent into combat. In this regard, Kohda and colleagues showed that blockade of the glucocorticoid receptor prior to exposure to single prolonged stress (SPS), an animal model of PTSD, prevented the development of enhanced fear responses in rats (Kohda et al. 2007). Also, blockade of the CRF1 receptor in a mouse predator stress model of PTSD prevented the initiation of stress effects (Adamec et al. 2010). However, there is an inherent risk for facilitating the development of PTSD with an intervention that inhibits glucocorticoid receptor signaling (Steckler, Risbrough 2011). This approach has up to date only been studied in a limited number of preclinical studies (Adamec et al. 2010, Kohda et al. 2007), and will require further investigation to develop appropriate prophylactic treatment strategies.

2.1.7.1.2 Secondary pharmacological prevention

Secondary pharmacological intervention refers to pharmacological manipulation of the stress response directly following the trauma. In this regard, many preclinical and clinical studies have been conducted in support of this strategy. Interestingly, opioids have shown potential in this category, with administration of morphine shortly after the trauma shown to reduce the likelihood of developing PTSD (Bryant et al. 2009, Holbrook et al. 2010, Saxe et al. 2001). Furthermore, the use of β-adrenergic antagonists has been suggested as a treatment option in the secondary prevention of PTSD after the trauma (Cahill et al. 1994, Reist et al. 2001, Vaiva et al. 2003). The α_2-receptor agonist, dexmedetomidine, was shown to block fear consolidation in rats (Adamec et al. 2010, Kohda et al. 2007) and mice (Davies et al. 2004), thus demonstrating the therapeutic potential of pre-synaptic α_2-receptor agonists in the secondary prevention of PTSD. However, stimulation of the presynaptic α_{2A}-adrenoceptor in two studies in humans showed no therapeutic effect (Davis et al. 2008, Neylan et al. 2006). NMDA-receptor antagonism was shown to interfere with anxiety-like behaviour in rats when administered shortly after exposure to predator stress (Adamec et al. 1998) and a retrospective study in a group of burn victims treated with the NMDA receptor antagonist, ketamine, during

hospitalisation showed a lower incidence of developing PTSD (McGhee et al. 2008). This would suggest that attenuating excitatory activity, or alternatively bolstering inhibitory GABA transmission, would be beneficial. However, this assumption is complicated by studies showing that treatment with the benzodiazepine, alprazolam (a GABA mimetic), enhances stress behaviour when administered after predator stress in rats (Matar et al. 2009). Moreover, human studies have confirmed these findings, with earlier studies showing that benzodiazepines facilitate memory for events immediately preceding administration (Hinrichs et al. 1984) and that secondary prevention of PTSD with benzodiazepines are ineffective, if not counter-effective (Gelpin et al. 1996, Mellman et al. 2002).

2.1.7.2 Treatment of non-cognitive symptoms of PTSD

The non-cognitive symptoms of PTSD include the emotional response, such as fearful reactions, hyperarousal, avoidance and numbing behaviour and failure of extinction of fear-related memories. The only currently approved pharmacotherapeutic treatment strategies are those that target these non-cognitive symptoms of PTSD and include various classes of antidepressants, adrenoceptor modulators, anticonvulsants and antipsychotics (Steckler, Risbrough 2011).

Various classes of antidepressants, such as the SSRIs, SNRIs, TCAs and MAOIs are employed in the treatment of non-cognitive symptoms of PTSD. The SSRI's have been shown to reduce symptom severity and to prevent relapse in PTSD patients (Brady et al. 2000, Connor et al. 1999, Martenyi et al. 2002, McRae et al. 2004, Van der Kolk et al. 1994), although only about 60% of patients respond to treatment with 20-30% of patients achieving full remission (Stein et al. 2002, Zohar et al. 2002). Moreover, the high degree of co-morbid major depression in many PTSD patients further complicates treatment and compromises a favourable outcome (Ravindran, Stein 2009). The other anti-depressants show a comparable efficacy to the SSRIs but, due to a less favourable side-effect profile, are not used as first line treatment (Bandelow et al. 2008). Anti-adrenergic agents such as prazosin have been studied in the

treatment of sleep-related disturbances in chronic PTSD, and presents with noteworthy efficacy in this regard (Raskind et al. 2003, Raskind et al. 2007, Taylor et al. 2008). With the growing awareness that PTSD involves a disturbance in excitatory vs. inhibitory transmitters (Harvey et al. 2004, MacKenzie et al. 2008), anticonvulsants have been proposed as an alternative treatment due to their putative anti-kindling effects. However, as noted above, amplifying GABA transmission with for example a benzodiazepine, may exacerbate PTSD symptoms (Gelpin et al, 1996).

2.1.7.3 Treatment of cognitive symptoms of PTSD

In PTSD, intrusive memories or flashbacks represent retrieval of aversive memories, which can subsequently be reconsolidated to strengthen the aversive memory trace (Charney 2004), a snowball-effect that results in strengthened fear memories with diminished context sensitivity. Preventing this reconsolidation process could lead to a weakened aversive memory trace. Glucocorticoid receptor agonists might be of use in preventing the constant reconsolidation of aversive memories (Taubenfeld et al. 2009, Tronel, Alberini 2007), while by the same token indirectly targeting NMDA-receptor function could also be of value, as NMDA-receptors play an important role in reconsolidation processes (Lee et al. 2006, Suzuki et al. 2004). Evidence for the role of β-adrenoceptor antagonists, such as propranolol, in the attenuation of fear memory reconsolidation demonstrates the potential value of these drugs in chronic PTSD (Brunet et al. 2008, Dębiec, Ledoux 2004).

Despite this broad armamentarium of drugs, for many patients treatment remains inadequate (Albucher, Liberzon 2002, Ravindran, Stein 2009, Schoenfeld et al. 2004). Indeed, treatment options for PTSD are severely limited by a lack of new and improved pharmacological treatments for the illness. This is driven primarily by a paucity of new and specific drug targets that will more effectively address the pathology of the illness. Consequently, a deeper knowledge of the underlying neurobiology of PTSD is needed, one in particular being pre-clinical studies that focus on the processing of fear memory

and its subsequent effect on key neuroendocrine mechanisms regulating the stress response.

2.2 LEARNING AND MEMORY

2.2.1 INTRODUCTION

Memory is defined as the ability to store, retain and retrieve information, while learning is the process by which these memories are formed. Memories can be categorised according to the content (declarative/explicit or procedural/implicit), duration (short-term or long-term memories) and nature of the memory (archival (long- or short-term) or transient (working) memory) (Izquierdo et al. 1999). Furthermore, memory processing is divided into distinct stages, namely acquisition, consolidation (and reconsolidation) and retrieval (Milner et al. 1998). To explain these stages, one might use an animal model of fear conditioning as an example. *Memory acquisition* occurs when the animal learns to associate the presentation of a cue light with an impending footshock. Therefore, an association between the presentation of the cue light and the receiving of a footshock is acquired and stored as a labile memory. *Consolidation* is a stage that can last from minutes to several days, and describes the process during which this labile memory is cemented into a more fixed state of memory, a process requiring protein synthesis (Davis, Squire 1984). Consolidation can also be described as the process by which short-term memory is transformed to long-term memory, making the memory increasingly resistant to further manipulation. *Reconsolidation* forms part of the consolidation process, but refers to when the stimulus is presented again to reinforce the memory. In a fear conditioning model, this would constitute repeated exposure of the animal to the cue-light and footshock pairings in multiple trials. In PTSD, flashbacks and memory intrusions often serve as reconsolidation of fearful memories, reinforcing the memory trace. *Retrieval* is the stage where the recollection of the aversive memory of the cue-light-

footshock-association takes place and is also the stage where memory retention can be determined (Abel, Lattal 2001).

Using behavioural training, the neural circuitry and associated biological processes underlying memory formation can be studied in animals. Shortly after a training task, memory processing takes place in the amygdala, medial septum and the hippocampus via activation of glutamatergic and cholinergic receptors. This process may be inhibited by $GABA_A$ receptors which are modulated by central β-noradrenergic and benzodiazepine receptors. Long-term potentiation (LTP) of the synapses activated during learning of the task is a crucial factor during memory processing (Izquierdo et al. 1993).

2.2.2 SYNAPTIC PLASTICITY AND LTP

Synaptic plasticity refers to the changes that take place in a synapse over a period of time that results in modification of synaptic strength. In 1973 Bliss and colleagues (Bliss, Lomo 1973) showed that brief, intense activation of a synapse results in the long-term potentiation of synaptic strength (Bliss, Lomo 1973). Simplified, synaptic plasticity is the modification of the strength of synaptic transmission. These modifications could be the result of altered numbers of receptors on the synapse, and this may be brought about by changes in the quantity of neurotransmitters released into the synapse (Gaiarsa et al. 2002). These long-lasting changes are thought to be an underlying mechanism of learning and memory.

LTP is a form of synaptic plasticity (Schafe et al. 2001) and is a widely accepted and popular model of the neurophysiological mechanisms proposed to be at the root of learning and memory formation. It refers to the long-term potentiation of synaptic transmission in the nervous system (Schafe et al. 2001). LTP has two distinct phases, namely early LTP (E-LTP) and late LTP (L-LTP) (Kandel 1997, Milner et al. 1998). E-LTP lasts for a few minutes and does not involve protein or RNA synthesis, only modifications of existing proteins. L-LTP can last for minutes to hours and even weeks and is dependent on protein and RNA synthesis (Schafe et al. 2001). The late phase of LTP can be seen as the consolidation phase, during which the synaptic strength is enhanced to

consolidate labile memories. A strong correlation between short- and long-term memory and E-LTP and L-LTP and their requirements for RNA and protein synthesis exists (Bourtchouladze et al. 1998, Grecksch, Matthies 1980), which suggests that memory consolidation can be understood at the cellular level through studies of LTP (Milner et al. 1998). Glutamatergic input is required for the hippocampal expression of L-LTP, and the activation of monoaminergic receptors plays a role in modulating these effects (Straube, Frey 2003). Studies in animals have shown that noradrenergic or β-adrenergic agonists can induce LTP in the dentate gyrus, which forms part of the hippocampal formation (Dahl, Sarvey 1989, Stanton et al. 1989) and that noradrenergic depletion and β-adrenergic antagonism can impair LTP in this structure (Bramham et al. 1997, Swanson-Park et al. 1999). Noradrenergic potentiation of glutamatergic inputs on LTP in the dentate gyrus was shown to be dependent on β-receptor activation (Caronolyn W. 2008). This correlates with the role of β-receptor activation in the consolidation of emotional memories, as shown in numerous studies employing fear conditioning (Cahill et al. 2000, Nielson et al. 1999, Roozendaal et al. 1997, Roozendaal et al. 1997, Vaiva et al. 2003, van Stegeren 2008). Indeed, many studies on the cellular mechanisms underlying fear conditioning investigated synaptic transmission and plasticity at excitatory inputs in the amygdala (Tsvetkov et al. 2004, Weisskopf, Ledoux 1999) promulgating the hypothesis that the rules governing synaptic plasticity and LTP also govern the rules of learning (to some extent) (Sigurdsson et al. 2007, Martin et al. 2000), and that LTP in the lateral amygdala mediates fear conditioning (Blair et al. 2001, Sigurdsson et al. 2007).

2.2.3 FEAR MEMORY

Fear is a defensive mechanism that helps an organism to recognise and appropriately react to threats in the environment. Fear can be seen as the activation of the defensive behavioural responses that serve to protect the animal or human from potential threats in the environment (Fendt, Fanselow 1999). Fear can be instinctive and unlearned, such as naïve laboratory-reared rats displaying fearful behaviour to feline urine in the predator scent stress animal model of PTSD (Kim, Jung 2006a). However, fear can also be learned,

and this is an important adaptive response to changes in the environment. This behavioural paradigm, where organisms learn to associate certain events or environments with a threat and consequently exhibit fearful responses, is modelled in laboratories in a paradigm called fear conditioning.

The fear response is marked by specific autonomic, endocrine and behavioural changes. Looking at an animal model of fear conditioning, an animal exposed to a predator or a stimulus reminiscent of a predator (such as a rat exposed to feline urine), will freeze (remain motionless), making no movements except for those required for respiration (Bolles, Collier 1976). Furthermore, the rat will display a fear-potentiated startle response (Brown et al. 1951, Davis et al. 1993), increased endocrine secretions of stress hormones, several autonomic changes (LeDoux 1992) and analgesia (Bolles, Fanselow 1980). In humans and animals these responses will abate as the threatening stimulus is removed, and the environment no longer holds any danger, but the memory processes consolidating the fearful situation into strong recollections of the event will go on for a much longer time, and in certain anxiety disorders, such as PTSD, this may result in maladaptive responses.

The *extinction* of fear memory (or of conditioned fear) is attained by multiple presentations of the conditioned stimulus in the absence of the unconditioned or aversive stimulus, resulting in reduced conditioned fear responses and reduced defensive behaviour (Meyers, Davis 2007, Tronson, Taylor 2007). This extinction is a very important adaptive response, and failure to extinct associations to stimuli that are no longer threatening is one of the symptoms commonly found in patients suffering from PTSD.

Acquiring fearful memories is an indisposable adaptive response; however, failure to “tune down” or extinct adaptive responses to cues that might no longer hold any threat, could eventually become unbeneficial and even be harmful to the organism. PTSD is a disorder of fear regulation where the learned fear responses to the original traumatic event are indiscriminately elicited in inappropriate situations, resulting in autonomic hyperarousal and a

generalized state of hyperarousal (Mahan, Ressler 2011). Fear conditioning is therefore employed to assist in elucidating the mechanisms resulting in a failure to "shut down" the fear response posttrauma.

2.3 FEAR CONDITIONING

2.3.1 DEFINITION

Fear conditioning is a behavioural paradigm using associative learning to study the neural mechanisms underlying learning, memory and fear (LeDoux 1992, Maren 2001). Fear conditioning occurs when a neutral stimulus (the conditioned stimulus or CS) is paired with an aversive stimulus (unconditioned stimulus or US), eliciting fearful responses to the neutral conditioned stimulus when presented even in the absence of the aversive or unconditioned stimulus (Rescorla 1968, Watson, Rayner 1920).

The "Little Albert experiment" performed by Watson and Rayner in 1920 is a well-known example of fear conditioning. When initially exposed to a white rat, Little Albert, an 11-month-old infant, responded with curiosity and playfulness toward the rat. When his hand touched the rat, the experimenters banged a steel bar with a hammer just behind the child's head, startling and frightening him and reducing him to tears. When the rat (CS) was presented to the child afterwards, Little Albert withdrew his hand and started wailing. Allegedly this fearful reaction was elicited towards other white, furry objects such as rabbits, muffs, soft toys etc. (Rescorla 1968).

In modern day studies, fear conditioning employs animals such as rats, mice or rabbits. These animals are presented with a neutral cue (CS) such as a cue light or tone, or a neutral context (such as an experimental chamber) together with an aversive unconditioned stimulus, such as a mild footshock. The animal learns to associate the neutral CS with the aversive US, producing conditioned fear responses (CR) (Maren 2001). In rats, typical CRs include freezing,

potentiated startle responses, decreased pain sensitivity and alterations in autonomic nervous system acitivity, such as increased pulse, blood pressure and respiration (Kim, Jung 2006a).

Conditioned fear learning occurs very rapidly and has a persistent effect. Therefore, fear conditioning is a valuable and popular tool for investigating the substrates of fear learning and memory (Davis 1997, LeDoux 2000).

2.3.2 TYPES OF FEAR CONDITIONING

2.3.2.1 Contextual Fear Conditioning

Contextual fear conditioning is the most basic of the conditioning procedures in which the essential role of the hippocampus (Diehl et al. 2007, Harooni et al. 2009) and amygdala (Kim et al. 1993, Phillips, LeDoux 1992) is implicated. It involves placing an animal in a novel environment (context) and presenting the animal with an aversive stimulus, such as a footshock, in this context. When the animal is returned to this context (usually 24 hours later), it should demonstrate a freezing response if the association between the context and the aversive stimulus is made (Wehner, Radcliffe 2004). Freezing is a species-specific response to fear, which has been defined as “absence of movement except for respiration”. This freezing response may last for seconds to minutes, depending on the strength of the aversive stimulus (i.e. intensity of footshock), the number of presentations (or number of trials), and the degree of learning achieved by the subject (Blanchard, Blanchard 1969, De Oca et al. 1998). Variations of contextual fear conditioning are step-down inhibitory avoidance and step-through inhibitory avoidance (passive avoidance) (Blanchard, Blanchard 1969).

2.3.2.1.1 Step-down inhibitory avoidance

In this paradigm, a rubber or woodend platform is placed on a grid floor which administers a footshock to the animal. The animal is placed on this “safe” platform, and when the animal steps down from this platform onto the grid floor, a footshock is administered. 24 hours later, the retention test is performed to

assess the animal's retention of the association between stepping down from the platform and the aversive stimulus (Martel et al. 2010, Martí Barros et al. 2004, Mello E Souza et al. 2000, Nasehi et al. 2010). An example of this paradigm is given in Figure 4.

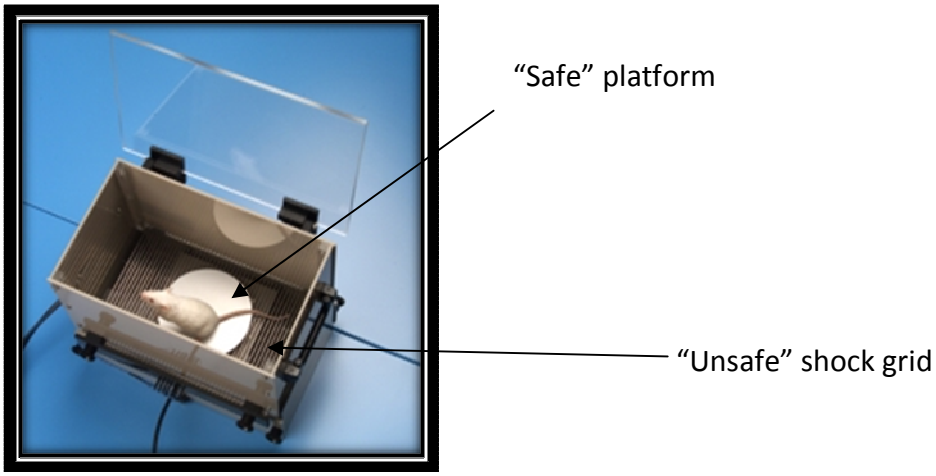

Figure 4: Example of a step-down inhibitory avoidance apparatus. The animal should learn not to step down from the platform in order to avoid a footshock.

Adapted from: http://www.myneurolab.com

2.3.2.1.2 Passive avoidance (Step-through inhibitory avoidance)

An example of an apparatus used in this paradigm is the Gemini™ Avoidance System, employed in this study (see Fig. 5). It consists of two chambers separated by an automated guillotine sliding gate. The floor of each chamber consists of steelrods 1 cm apart through which footshocks can be delivered. This task uses the animal's natural preference for a dark area to test aversive learning and subsequent behavioural adaptation (Porciúncula et al. 2002). In this task, subjects are placed in the one chamber with the guillotine gate closed (day one). This chamber is usually illuminated during or after habituation. After a certain period, the sliding gate opens and the animal is free to move across to the dark chamber, where upon entrance the gate closes and a mild footshock is administered to the subject. The subject should learn to associate this preferred dark context with an aversive stimulus, and should subsequently display an aversion to enter this compartment. The retention test is conducted 24 hours

later (day two). The latency to cross through the gate in order to avoid the stimulus is used as an indicator of learning and memory – a higher latency (time) indicates learning and memory for the aversive association between the context and footshock (Ohno, Watanabe 1996).

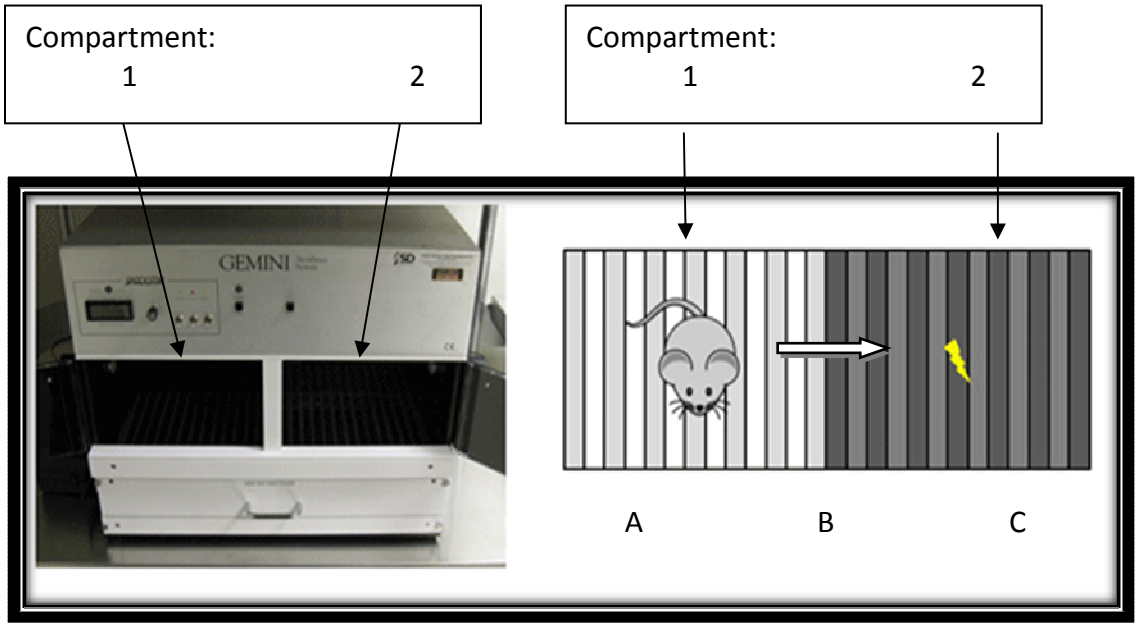

Figure 5: Photo of the Gemini™ Avoidance System (left) with a schematic representation of the passive avoidance procedure (right). (A) The animal is placed into compartment 1, which is illuminated or lights up after a few seconds. (B) The gate opens after a predetermined time, and the animal is free to cross over to the preferred dark environment in compartment 2. (C) Upon entrance in the dark compartment, the door closes and the animal is presented with a footshock. For a detailed description please refer to the text in section 2.3.2.1 under *Passive Avoidance (Step-through inhibitory avoidance).* Adapted from: http://sbfnl.stanford.edu/bml_passive.html

2.3.2.2 Cued Fear Conditioning

Cued fear conditioning is similar to contextual conditioning, with the exception that a CS, such as a cue light or a tone is added to the context, serving as the predictor of the aversive event, rather than the context. In this paradigm, the animal should learn that the presentation of a cue light or tone (or both) predicts an aversive stimulus such as a footshock, and the animal should then escape to the opposite chamber if adequate learning of the association was acquired. An example of cued fear conditioning is active avoidance (see Fig. 6). In

contrast to contextual fear conditioning, cued fear conditioning is dependent on the amygdala (Kim, Jung 2006a, Phillips, LeDoux 1992). In order to separate context from cue conditioning, some investigators employ a pre-exposure trial to the context without presenting the US, which is usually a footshock. This then allows the animal to take in all the information about the context without associating it with an aversive event or the presentation of a cue. On a second exposure to the context, the CS, such as a tone or cue light is presented and the animal is better able to learn the CS association because the context is not as accurate a predictor of shock as the CS. A distinction between trace cued fear conditioning and delay cued fear conditioning can be made (Curzon et al. 2009).

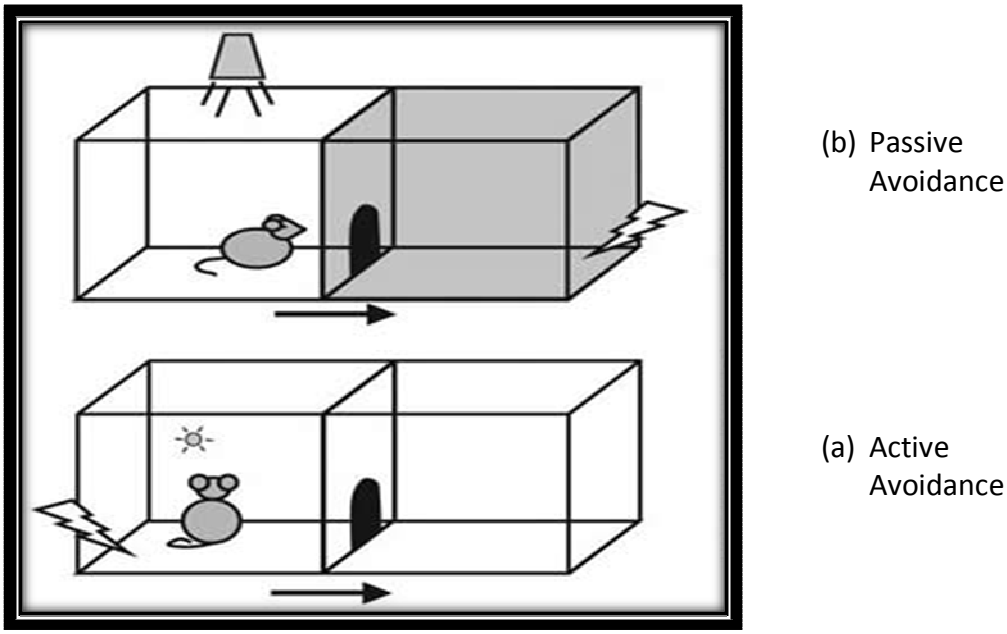

Figure 6: Graphical representation of the difference between (a) passive avoidance and (b) active avoidance protocols: passive avoidance (a) exploits a natural tendency of the animal to prefer dark environments. In active avoidance (b) the animal learns to avoid the administration of the footshock (US) based upon the presentation of a cue (CS) which can be a tone or a cue light. Adapted from http://tech.groups.yahoo.com/group/Medical_Physiology/message/228

2.3.2.2.1 Delay Fear Conditiong

In delay fear conditioning, the CS is paired with the US so that the two stimuli co-terminate (see Fig. 7B). Both memory for contextual and cued stimuli can be measured (Gould et al. 2004).

2.3.2.2.2 Trace Fear Conditioning

Trace conditioning (see Fig. 7C) differs from delay conditioning in that the US is presented after a certain time interval, therefore the CS and US do not co-terminate. Trace conditioning uses additional brain regions in order to establish the response (see section 2.3.3.1 and 2.3.3.2.). Depending on the particular region of interest under investigation, researchers should decide on the appropriate testing paradigm. Relatively small differences in associative learning are observed between trace and delay conditioning when the trace interval is short (2-5 seconds). However, the longer the trace interval becomes (45-60 seconds have been used), the weaker the association to the cue becomes, and multiple repeated training trials are required in order for the association between the CS and US to be formed (Curzon et al. 2009).

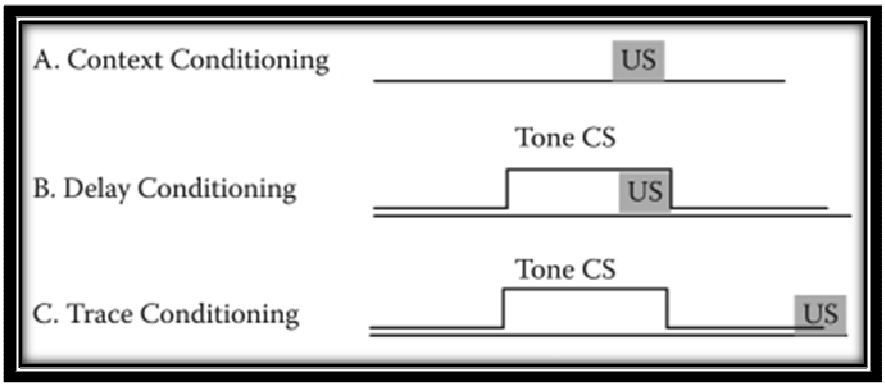

Figure 7: Graphical representation of the order of presentation of CS and US in different types of fear conditioning. In contextual fear conditioning (A), such as passive avoidance, no CS is given and the US is presented after a predetermined exposure to the context. In delay fear conditioning (B), such as active avoidance, the CS (a tone or cue light) is presented, and after a predetermined time the US is simultaneously presented, co-terminating with the CS. In trace fear conditioning (C), the CS is presented, terminated and after a predetermined amount of time has elapsed, the US is presented. Adapted from http://www.ncbi.nlm.nih.gov/books/NBK5223/

2.3.3 Neuroanatomy of Fear Conditioning

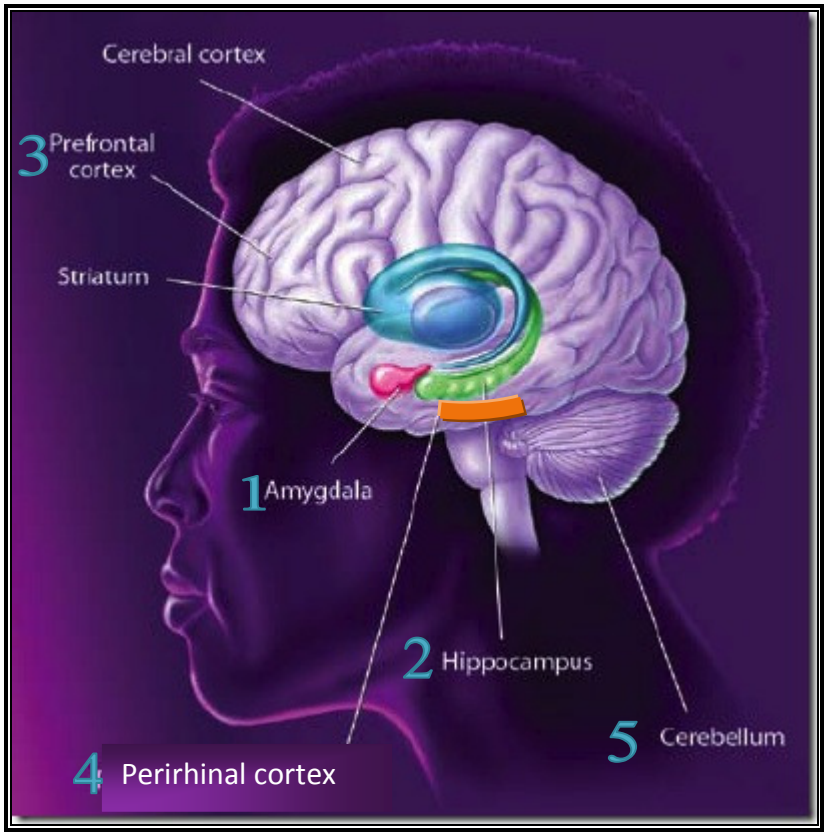

Figure 8: Brain regions involved in fear conditioning. The brain regions discussed in this dissertation are numbered in order of their appearance under section 2.3.3: 1) The Amygdala, 2) The Hippocampus, 3) The Prefrontal Cortex, 4) The Perirhinal cortex, 5) The Cerebellum. Adapted from:http://www.militaryhealthmatters.org/wpcontent/uploads/2011/08/image_thumb18.png

2.3.3.1 The Amygdala

The amygdala (see Fig. 8 and Fig. 9) has been implicated as the site of fear conditioning, drawing from extended evidence from lesion, neurophysiological and pharmacological studies (Davis 1997, Fendt, Fanselow 1999, Kim, Jung 2006a, Lavond et al. 1993). Lesions to the lateral amygdala have been shown to inhibit the acquisition of fear, as well as the expression of previously acquired fear (LeDoux et al. 1990a, Maren et al. 1996, Muller et al. 1997). Figure 15 on page 59 offers a schematic representation of reciprocal connections between the amygdala and other brain areas to mediate the fear response. CS and US inputs from the thalamus (LeDoux et al. 1984, LeDoux et al. 1990b) and cortex

(Romanski, LeDoux 1993) both reach the basolateral nuclei of the amygdala (BLA), where response of individual neurons is generated (Romanski et al. 1993), suggesting convergence of these inputs at the lateral nuclei of the amygdala (LA). Therefore, the association between the CS and US is believed to be formed in the BLA, hence the belief that the amygdala underlies fear conditioning (Kim, Jung 2006b). This is believed to be mediated by an increase in synaptic strength (synaptic plasticity) between the synapses transmitting CS input to the neurons in the BLA (Blair et al. 2001, Rogan, Ledoux 1995, Rogan et al. 1997). The BLA then relays this information to the central nucleus of the amygdala (CeA) which, via projection neurons to the hypothalamus and brainstem, is responsible for the expression of fear by behavioural (startle or freezing response), autonomic and endocrine responses (Davis 1997, Kim, Jung 2006a, LeDoux et al. 1988, Sigurdsson et al. 2007). However, it remains to be elucidated whether the amygdala is the site of permanent storage of long-term fear memory (Kim, Jung 2006b).

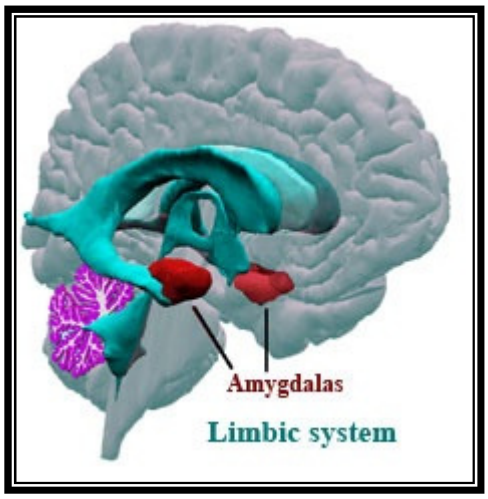

Figure 9: The amygdala, an integral part of the limbic system. Adapted from: http://co-bw.com/STW/Images_STW/amygdala2.JPG

2.3.3.2 The Hippocampus

Evidence for the involvement of the hippocampus (see Fig. 8 and Fig. 10) in certain types of fear conditioning is also available. Hippocampal lesions have been shown to impair trace fear conditioning to an auditory cue, but not delay fear conditioning to the same cue (McEchron et al. 1998, McEchron et al. 2000)

(please refer to section 2.3.2.2 for an explanation of trace and delay fear conditioning). Furthermore, it seems that the hippocampus has a transient role in storing contextual fear memory. Selective lesions of the hippocampus 1 day after contextual fear conditioning in rats resulted in abolishment of the conditioned fear. However, when a delay of 28 days was imposed between contextual conditioning and hippocampal lesioning, contextual conditioned fear remained considerably intact (Anagnostaras et al. 1999, Kim, Fanselow 1992, Maren et al. 1997). Furthermore, lesions to the nucleus accumbens, which receives efferent input from the hippocampus, resulted in obstruction of contextual fear conditioning, but not auditory fear conditioning (Riedel et al. 1997). Thus, the hippocampus is important, if only transiently, in contextual fear conditioning, and also plays a role in auditory and trace, but not delayed fear conditioning.

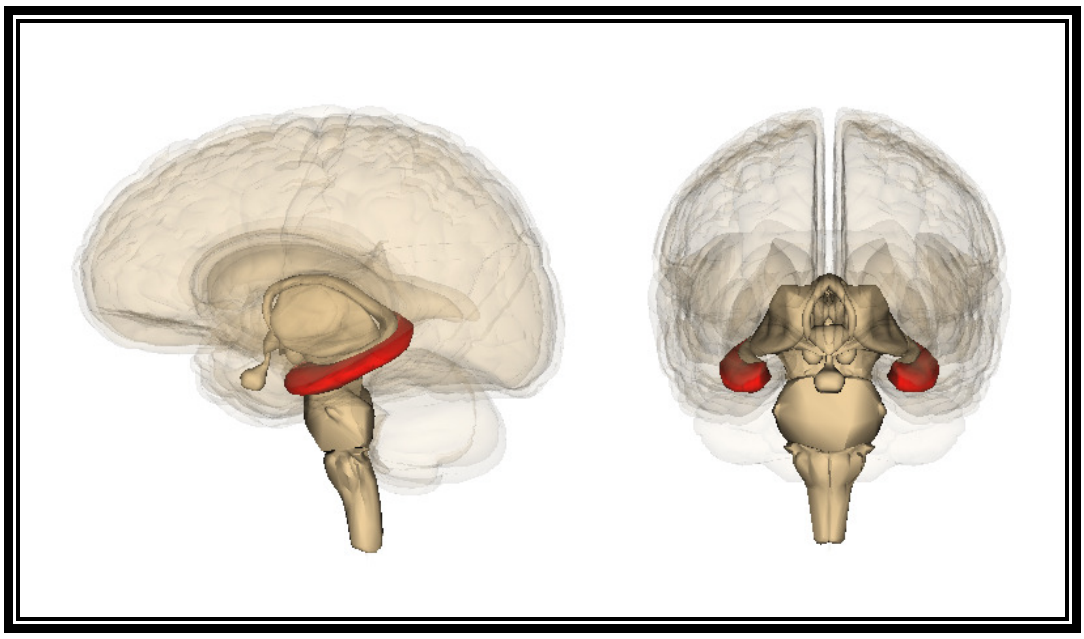

Figure 10: The hippocampus (indicated in red), situated in the medial temporal lobe. Adapted from http://www.psypost.org/wp-content/uploads/2010/11/Hippocampus.png

2.3.3.3 The Prefrontal Cortex (PFC)

The prefrontal cortex (see Fig. 8 and Fig. 11) projects to the hippocampus and amygdala, maintaining inhibitory control over these structures and over maladaptive behaviour, and is important in the extinction of conditioned fear

responses (Morgan et al. 1993b). When the CS reappears after the passage of time without simultaneous presentation of the US, it is no longer necessary or beneficial for the animal to exhibit the previously adaptive behavioural responses of fear to the CS. Studies in animals showed that stimulation of the PFC results in reduced conditioned fear responses and decreased neuronal firing from the amygdala (Milad, Quirk 2002, Milad et al. 2004, Quirk et al. 2003, Rosenkranz, Grace 2001, Rosenkranz, Grace 2002). Furthermore, studies employing models of fear extinction (where an animal is trained to unlearn a conditioned response to a stimulus previously paired with an unconditioned aversive stimulus, see section 2.2.3) suggested the ventromedial PFC (vmPFC) as an important facilitator of fear extinction (Baeg et al. 2001, Morgan et al. 1993b, Phelps et al. 2004, Santini et al. 2004). Behavioural studies investigating the role of the PFC in extinction of conditioned fear are however not unanimous in their findings, with many of them conflicting. Although a large body of evidence points to the PFC as an important regulator of fear extinction, further probes into this field of study are required (Kim, Jung 2006a).

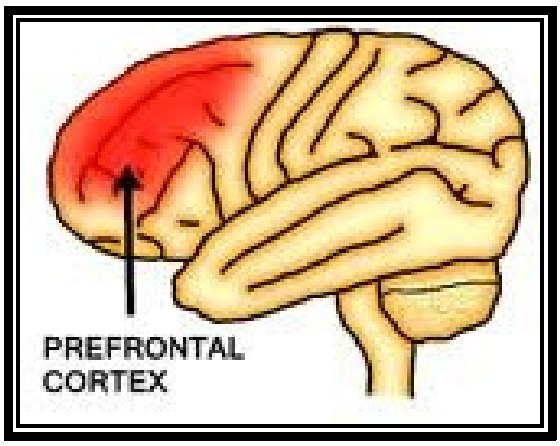

Figure 11: The PFC. Adapted from:
http://www.mmtheory.com/preliminary/prefrontal%20cortex.jpg

2.3.3.4 The Perirhinal Cortex

The perirhinal cortex (see Fig. 8 and Fig. 12) is located in the medial frontal lobe and receives processed sensory information from all sensory regions. It is reciprocally connected to the hippocampus and some evidence suggests a role in the early consolidation and/or storage of hippocampus-dependent contextual

memory (Kim, Jung 2006a). Lesions of the perirhinal cortex made one day after training in a contextual fear conditioning paradigm, resulted in decreased retention of contextual fear memory (Burwell et al. 2004).

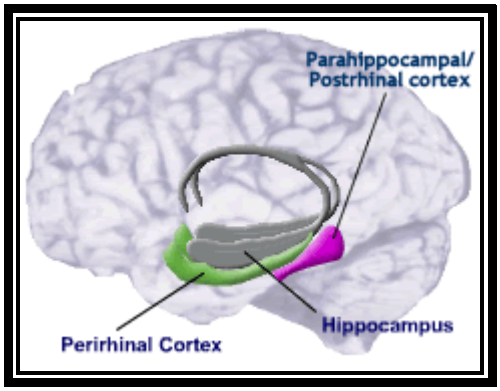

Figure 12: The Perirhinal cortex, located in the medial temporal lobe and bordered caudally by the parahippocampal region. Adapted from: http://www.bristol.ac.uk/synaptic/pathways/

2.3.3.5 The Cerebellum

The cerebellar vermis is also implicated in the neural circuitry underlying fear conditioning. Supple and colleagues showed that lesions of the cerebellar vermis abolished conditioned autonomic responses, attenuated freezing and decreased anxiety-like behaviour in rats (Supple Jr., Leaton 1990a, Supple Jr., Leaton 1990b, Sacchetti et al. 2002). These findings suggest a role for the cerebellar vermis in fear conditioning and fear-related behaviour. See Figure 8 and Figure 13 for the location of the cerebellum.

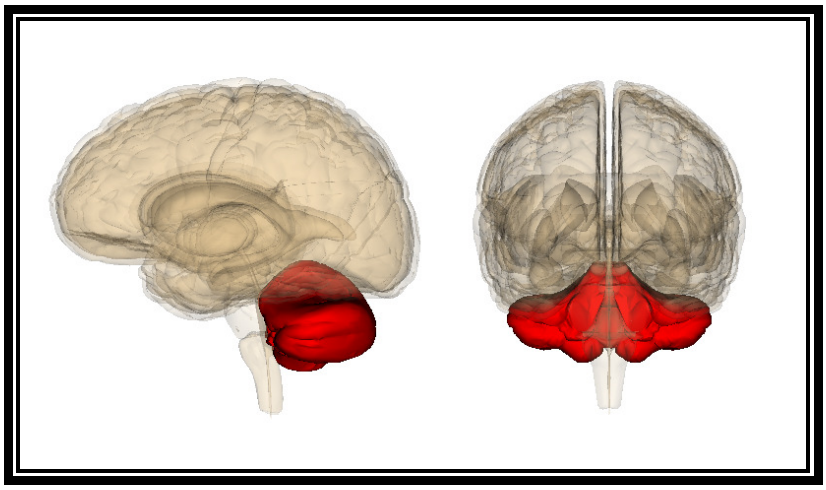

Figure 13: The cerebellum. Adapted from: http://en.wikipedia.org/wiki/File:Cerebellum.png

2.3.3.6 The Insular Cortex

The insular cortex (see Fig. 14) is a part of the cerebral cortex which is found deep within the lateral sulcus and lies between the temporal and frontal lobes. This structure is important in diverse functions, usually related to emotion or homeostasis, and includes cognitive functioning, self-awareness, perception, motor control and interpersonal experience. Sensory information is relayed between the insular cortex and amygdala (Turner, Zimmer 1984) and this structure might have a role in the storage of fear memory (Kim, Jung 2006a). Rosen and colleagues showed impaired retention of the fear-potentiated startle response when lesions to the distal aspect of the insular cortex were made in a study using a visual conditioned stimulus (Rosen et al. 1992), and inactivation of the insular cortex impaired retention in an inhibitory avoidance paradigm of fear conditioning (Bermudez-Rattoni et al. 1991). Due to its reciprocal connection with the amygdala and its role in emotion and cognition, the insular cortex could be implicated in fear conditioning neurocircuitry (Kim, Jung 2006a).

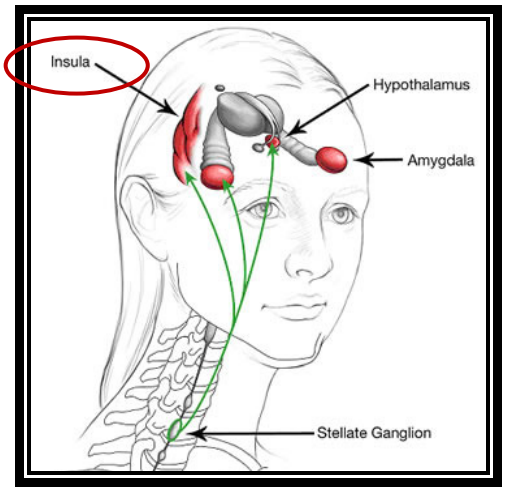

Figure 14: The insular cortices (insula), located deep within the cerebral cortex, between the temporal and frontal lobes. Adapted from: http://www.hotflashescure.com/mechanism.html

2.3.3.7 Conclusion

The neural circuitry underlying fear conditioning is far from simple, and the structures implicated in fear conditioning are reciprocally connected so that no single structure is solely responsible for the acquisition, consolidation or

extinction of conditioned fear. Understanding the neurocircuitry underlying fear conditioning as well as how this relates to the neurobiology proposed to facilitate fear conditioning, is useful in forming a picture of how each of these structures connect to orchestrate the vast processes that ultimately serve to produce adaptive responses to the environment.

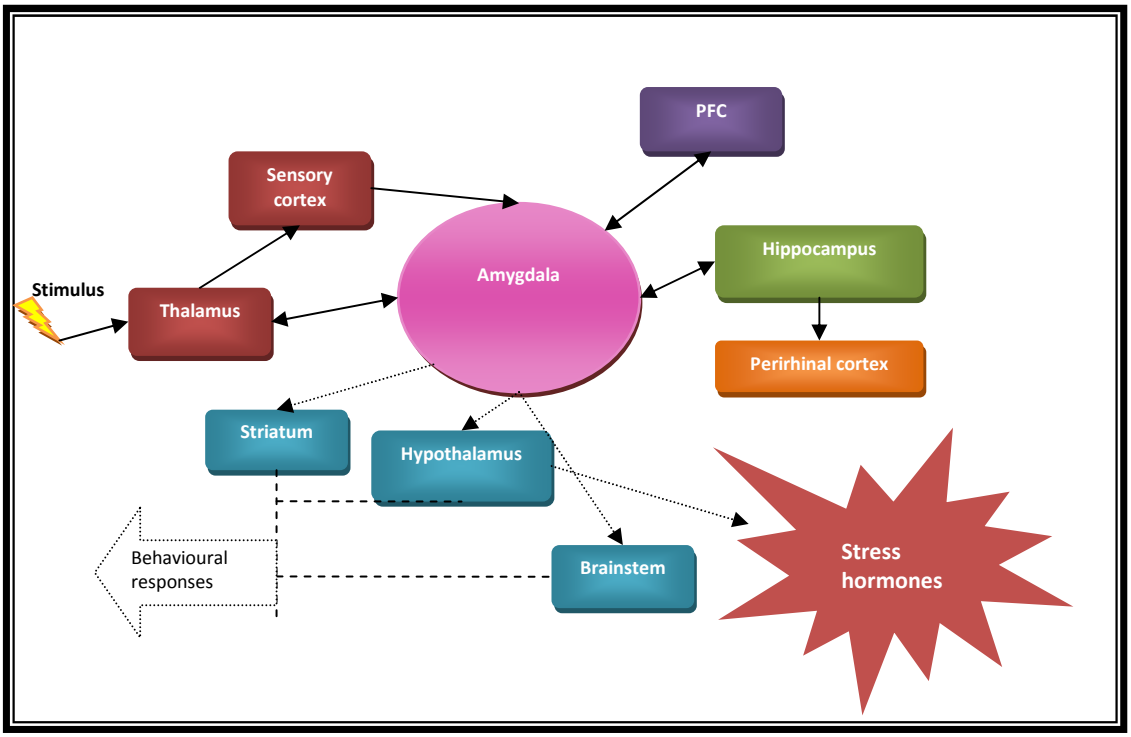

Figure 15: Involvement of different brain areas in emotional learning and memory. A stimulus is carried from the thalamus and sensory cortices to the amygdala, which integrates the information and orchestrates emotional responses via the striatum, hypothalamus and brainstem (dashed lines represents output of behavioural responses). The information is relayed to the PFC and hippocampus and the hippocampus processes contextual information along with the perirhinal cortex. The PFC and hippocampus exert behavioural and contextual control over the amygdala

2.3.4 The Neurobiology of Fear Conditioning

2.3.4.1 Cholinergic signaling

Acetylcholinergic processes have long been implicated in learning and memory, and a specific role of acetylcholine in hippocampus-dependent learning processes has been suggested (Gould et al. 2004, Hasselmo et al. 1996).

Muscarinic and nicotinic processes may facilitate learning and memory via hippocampal cholinergic neurons, and these effects are seen in the retrieval of long term memory, as well as in the formation and maintenance of short-term working memory (Mizoguchi et al. 2001). Infusions of muscarinic receptor antagonists into the hippocampus (Grette Lydon, Nakajima 1992b), PFC (Broersen et al. 1995, Broersen 2000) and amygdala (Bianchin et al. 1999) have all been shown to have detrimental effects on memory and cognitive functioning. Gould and colleagues demonstrated that nicotine enhances hippocampus-dependent fear conditioning via cholinergic pathways (Gould et al. 2004). Interestingly, Brand and colleagues recently found that animals subjected to time dependent sensitisation (TDS) stress, an animal model of PTSD, demonstrate an increase in sensory-mediated aversive memory. This phenomenon is akin to the flashbacks experienced in patients with PTSD following a brief sensory recall of the traumatic event. More important, however, is that they show that this response involves cortico-limbic cholinergic M_1-receptor-driven pathways (Brand et al. 2008).

2.3.4.2 Glutamatergic signaling

Glutamate is the main excitatory neurotransmitter in the brain and is essential in the consolidation and extinction of conditioned fear. Glutamatergic transmission in the BLA was shown to occur after fear conditioning in rats (Lin et al. 2010). Glutamatergic neurons project from the sensory thalamic and cortical structures, the hippocampus and PFC to the BLA (Pape, Pare 2010) and the BLA in turn transmits glutamatergic signals to the central amygdala. Glutamate receptors can be divided into ionotropic (NMDA, AMPA) and metabotropic (mGluR 1-8) receptors, all of which have been implicated in fear conditioning (Mahan, Ressler 2011). The ionotropic glutamate receptors are important in the mediation of synaptic plasticity for the formation of long-term fear memories, whilst metabotropic receptors modulate synaptic plasticity via G-protein coupled signal transduction (Rumpel et al. 2005). Current evidence suggests that fear conditioning activates NMDA receptors, causing a cascade of signal transduction mechanisms which result in the synaptic upregulation of AMPA receptors, which in turn results in LTP and enhanced responsiveness of

the synapse to future presentations of the conditioned stimulus (Nedelescu et al. 2010, Rumpel et al. 2005). Translational animal models of PTSD have also demonstrated robust involvement of various aspects of glutamate signaling (see Harvey and Shahid 2011 for review), including that of the nitric oxide pathway (Harvey et al. 2004, Harvey et al. 2005, Harvey et al. 2005, Harvey, Shahid 2011, Oosthuizen et al. 2005).

2.3.4.3 GABAergic inhibitory regulation

GABA is the main inhibitory neurotransmitter in the central nervous system, and GABAergic transmission is also thought to be important in the precise regulation of fear conditioning (Makkar et al. 2010, Zhang, Cranney 2008). Rea and colleagues found reduced GABAergic signaling in the basolateral nucleus of the amygdala in fear-conditioned subjects relative to non-fear conditioned controls (Rea et al. 2009). Moreover, GABAergic inactivation of the PFC, amygdala and hippocampus, as well as certain striatal regions was shown to impair various aspects of fear conditioning (Raybuck, Matthew Lattal 2011, Sierra-Mercado et al. 2011). Pronounced GABAergic activity would therefore lead to diminished consolidation, expression and extinction of conditioned fear (Mahan, Ressler 2011). Important to note is that TDS, a putative animal model of PTSD, attenuates GABA levels and alters glutamate NMDA receptor binding in the rat hippocampus (Harvey et al. 2004). Thus, the combined change in glutamatergic and GABAergic signaling plays a profound role in the fear response, while the change in excitatory versus inhibitory transmission in the brain may explain the structural brain changes evident in patients with PTSD (Oosthuizen et al. 2005).

2.3.4.4 The role of glucocorticoids

Glucocorticoids are released from the adrenal cortices when the HPA axis is activated as part of the biological stress response to emotionally arousing experiences, and the role of these stress hormones in facilitating long-term memory consolidation have been well-studied (De Kloet et al. 1999, McGaugh, Roozendaal 2002, Roozendaal 2000). In contrast, glucocorticoids do not appear to be essential for memory consolidation of neutral or non-emotionally-

loaded events or information (Buchanan, Lovallo 2001, Okuda et al. 2004). When glucocorticoids are administered directly after a fear conditioning training task, memory consolidation for the task is enhanced in a fashion closely related to the effect of post-training noradrenaline administration (Cordero, Sandi 1998, Pugh et al. 1997, Roozendaal et al. 1999, Hui et al. 2004). Furthermore, post-training administration of glucocorticoids have proved their role in memory consolidation in auditory-cue fear conditioning (Hui et al. 2004, Zorawski, Killcross 2002) and in Pavlovian appetitive discrete-cue conditioning (Zorawski, Killcross 2002). Extensive research has produced unanimous evidence for the necessity of noradrenergic co-activation in the BLA as a requirement for glucocorticoid modulation of memory consolidation (Quirarte et al. 1997, Quirarte et al. 1998, Roozendaal et al. 1999, Roozendaal et al. 2002). Furthermore, the corticosterone synthesis inhibitor, metyrapone, blocked enhancement of passive avoidance retention induced by post-training administration of adrenaline (Roozendaal et al. 1996). Noradrenergic mechanisms are therefore crucial for glucocorticoid-induced memory consolidation of emotionally arousing events.

Glucocorticoids not only facilitate emotional memory consolidation, but are also implicated in the impairment of spatial and contextual memory retrieval following stressful events (De Quervain et al. 1998). A high density of glucocorticoid receptors (GRs) are present in the hippocampus (Reul, De Kloet 1985) and modulation of these receptors in aversive and appetitive learning tasks have shown definite effects on memory consolidation. These effects have been shown to be dependent on activation of the amygdala (Roozendaal, McGaugh 1996, Roozendaal 2000, Roozendaal 2002) while evidence for the critical involvement of the BLA in glucocorticoid-dependent emotional memory consolidation has also been shown (Roozendaal, McGaugh 1996).

Glucocorticoids act through intracellular and intranuclear GRs and may affect gene transcription through binding of receptor homodimers to DNA (Beato et al. 1995, Datson et al. 2002). Glucocorticoids are also implicated in increasing phosphorylation of extracellular regulated kinase (ERK2), which is a subtype of

mitogen-activated protein kinases. ERK2 is considered to be critical for long-term neuronal plasticity and memory consolidation in the amygdala and hippocampus, and can be activated by noradrenergic stimulation resulting in the formation of cyclic adenosine monophosphate (cAMP) (Impey et al. 1999, Schafe et al. 2000). In Fig. 16 the role of glucocorticoids in the synthesis of proteins for memory consolidation and the intricate involvement of the noradrenergic system is illustrated. Glucocorticoids acting on GRs in the nucleus of the solitary tract (NTS) potentiate presynaptic noradrenergic release in the BLA, while glucocorticoids acting on GRs in the BLA facilitate the noradrenergic activation of the cAMP/PKA (cAMP-dependent protein kinase) signaling cascade (see section 2.3.4.5), resulting in protein synthesis, which modulates memory consolidation in the hippocampus and other brain areas (McGaugh, Roozendaal 2002). The role of glucocorticoids in the neurobiology of fear conditioning is well-established and the involvement of noradrenergic mechanisms in the activation of glucocorticoid-mediated memory consolidation is of great relevance for studies into noradrenergic mechanisms in the neurobiology of fear.

Turning briefly again to PTSD, studies in animals submitted to a TDS stress paradigm have found that stress-induced changes in glutamate-nitric oxide signaling is attenuated by ketoconazole, a glucocorticoid synthesis inhibitor (Harvey et al 2004), while early life stress and later re-stress alters HPA axis function together with a decrease in glucocorticoid receptor levels in the dentate gyrus of the hippocampus (Uys et al. 2006). Moreover, a number of notable animal models of PTSD, such as TDS and single prolonged stress (SPS), induce hypocortisolemia in animals subjected to stress (see Harvey and Shahid 2011 for review). This is also a typical neuroendocrine anomaly seen in patients with PTSD and which may have relevance for explaining the diverse changes in implicit, explicit and fear-related memory function in the disorder.

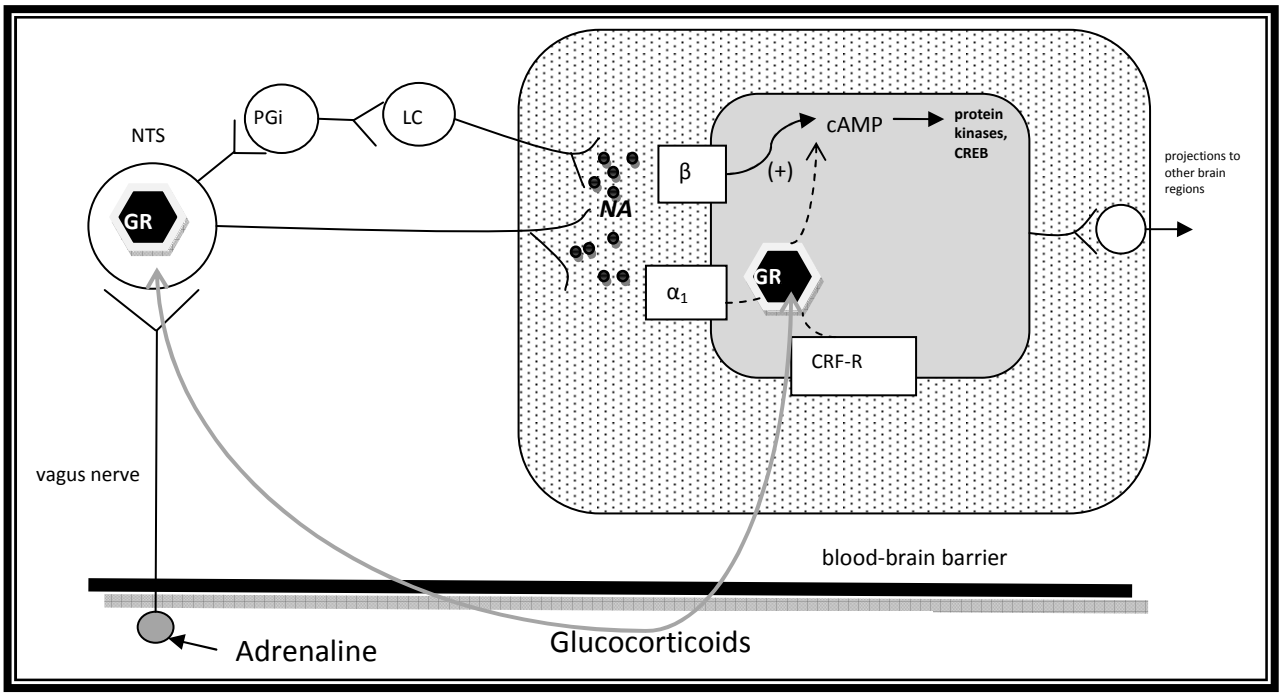

Figure 16: Schematic representation of the glucocorticoid and noradrenergic mechanisms regulating memory consolidation in fear conditioning within the amygdala. During the training trial, adrenaline and glucocorticoids are released from the adrenal cortices. Adrenaline does not cross the blood-brain barrier, but activates the vagal afferents to the NTS. Noradrenergic neurons in the NTS project directly into the BLA and indirectly via the PGi to the LC. Activation of the vagas nerve by adrenaline causes these projections to release NA into the BLA from the LC and the NTS. NA then binds postsynaptic β- and α_1-adrenoceptors in the BLA, which facilitates the NA signaling cascade involving cAMP, resulting in the production of PKA and CREB, eventually facilitating protein synthesis. Glucocorticoids released from the adrenal cortices cross the blood-brain barrier and bind to GRs in the NTS to potentiate NA release in the BLA, and to postsynaptic GRs in the BLA to facilitate the NA signal transduction pathway. The effects of adrenaline and glucocorticoids on NA activation in the BLA are required for regulating memory consolidation in other brain regions. CRF may facilitate the β-receptor mediated cAMP response cascade by acting on the GR that is independent of α_1-induced modulation. Therefore, α_1-receptor induced and CRF-R induced GR potentiation synergystically potentiate training induced β-receptor activation of the cAMP pathway. Adapted from De Quervain et al, 2009 and McGaugh et al 2002.

Abbreviations: α_1= alpha-1-adrenoceptor; β= beta-adrenoceptor; cAMP=adenosine 3',5'-cyclic monophosphate; CREB= cAMP response-element binding; PGi = nucleus paragigantocellularis; CRF-R = corticotropin-releasing factor receptor.

2.3.4.5 Noradrenergic signaling

Upon receiving aversive unconditioned stimuli, the stimuli is transmitted to the spinal cord, which can follow a number of different pathways to transmit the stimuli into the amygdala, one being the nucleus paragigantocellularis (PGi), which relays the stimuli to the locus coeruleus (LC) and from there the stimulus can be forwarded to the BLA (Fendt, Fanselow 1999) (see Fig. 16). As discussed in section 2.1.7.1, a vast amount of noradrenergic neurons project from the LC to the amygdala, hippocampus and the mPFC. The noradrenergic system is therefore an important mediator of the fear response and the subsequent laying down of strong fearful memories. Experiences that elicit strong emotional responses are transformed into very strong memories, more so than neutral experiences (McGaugh 2002), and these experiences are associated with increased noradrenergic levels in the brain areas involved in learning and memory (McGaugh et al. 2002, McGaugh 2004, McIntyre et al. 2002). Soeter and colleagues found that stimulating the noradrenergic system during memory formation strengthens the fear potentiated startle response in humans (Soeter, Kindt 2011) and emotionally aroused noradrenergic activation of the BLA has been shown to strengthen memory consolidation in other brain regions such as the hippocampus (McGaugh 2004). A large body of evidence supports the role of noradrenergic receptor activation in the amygdala as a crucial mediator for memory consolidation. Importantly, these studies employed β-receptor antagonism in the amygdala to produce inhibition of memory consolidation, and β- and α-receptor agonism in the BLA to produce enhancement of memory consolidation (Ferry et al. 1999a, Ferry et al. 1999b, Ferry, McGaugh 1999, Liang et al. 1986, Liang et al. 1990). Furthermore, administration of a footshock to rodents, such as is used in passive avoidance paradigms (also known as inhibitory avoidance), induces the release of noradrenaline in the amygdala which is directly correlated to the intensity of the shock (Galvez et al. 1996, Quirarte et al. 1998) and to the measure of retention (McIntyre et al. 2002).

Noradrenergic involvement in memory processes studied in fear conditioning is illustrated in Fig. 16. Adrenaline is released from the adrenal cortices in

response to fear conditioning tasks. Adrenaline induces the release of noradrenaline by activating vagal afferents to the NTS. Noradrenergic neurons in the NTS project directly to the BLA as well as indirectly, via the PGi which projects to the LC. The latter in turn projects to the BLA. Noradrenaline released from the NTS and the LC activates postsynaptic β and α_1-receptors in the BLA. Activation of these receptors activates the cAMP pathway resulting in cAMP-dependent protein kinase (PKA) formation (McGaugh, Roozendaal 2002). PKA is implicated in LTP in the amygdala (Huang, Kandel 1998). Activation of PKA activates the transcritpion factor cAMP-response element-binding protein (CREB) (Carew 1996), an important factor in long-term memory formation for fear conditioning (Josselyn et al. 2001).

The role of glucocorticoid and glutamatergic mechanisms in fear conditioning has been discussed in the aforegoing sections. However, it is important to note that noradrenergic activation within the BLA is thought to be necessary in the modulation of these mechanisms (McGaugh, Roozendaal 2002). Noradrenergic activation in the BLA was shown to activate glutamatergic mechanisms in this structure and to facilitate NMDA-dependent plasticity (Huang et al. 1994, Lennartz et al. 1996, Wang et al. 1996). Furthermore, Roesler and colleagues showed that an NMDA-antagonist induces memory deficits in rats which could not be reversed by noradrenaline, suggesting that the modulatory effects of noradrenaline on memory consolidation involve NMDA-dependent mechanisms (Roesler et al. 1999).

Noradrenergic cell groups in the NTS and the LC express high densities of glucocortoid receptors (Harfstrand et al. 1986). Quirarte and colleagues found that the memory enhancement induced by post-training dexamethasone administration was blocked by infusion of a β-adrenoceptor antagonist into the BLA (Quirarte et al. 1998). Similarly, Roozendaal and colleagues showed a dose-dependent enhancement of memory when glucocorticoid receptors on noradrenergic cell groups in rat NTS were activated after a training task. This enhancement was blocked by subsequent infusion of a β-receptor antagonist into the BLA (Roozendaal et al. 1999).

These findings clearly suggest that noradrenergic modulation in the BLA is vital in both glucocorticoid and glutamatergic mechanisms underlying memory

consolidation. The noradrenergic system is therefore an important target of investigations into the neurobiology of disorders of stress and memory, such as PTSD.

2.3.5 APPLICATION OF FEAR CONDITIONING IN STUDIES OF STRESS AND ANXIETY DISORDERS

2.3.5.1 Fear conditioning as tool for investigating CNS cognitive processes

Generalised anxiety disorder, panic disorder, obsessive compulsive disorder (OCD), social phobia and PTSD are all anxiety disorders that are related to some or other dysfunctional processing of fear and fear-related processes (American Psychiatric Association 2000)(Sandford et al. 2000). Fear conditioning is used to study the neurobiology, neurocircuitry and aetiology of these disorders, and to investigate possible treatment approaches (De Oca et al. 1998, Kim et al. 1993, Kim, Jung 2006a). For the purpose of the current study, however, only its application in PTSD will be discussed.

2.3.5.2 Application of fear conditioning in PTSD

PTSD is associated with impaired PFC function and excessive amygdala activity (see section 2.1.5), as well as impaired hippocampal function, a vital brain area in consolidation of contextual fear memories (Karl et al. 2006, Shin et al. 2006). Therefore, patients with PTSD or animals in an animal model of PTSD should elicit stronger conditioned fear responses and impaired extinction of fear in the absence of the unconditioned stimulus. Indeed, PTSD includes symptoms that show impaired extinction of fear memories (flashbacks, nightmares, memory intrusions) and a tendency to show exaggerated conditioned fear responses (exaggerated startle response, etc) (American Psychiatric Association 2000), while contemporary theories of PTSD concur that learning and memory processes such as fear conditioning underlie these symptoms (Michael et al. 2005, Pitman 1989, Rothbaum, Davis 2003). It is

therefore valuable to perform fear conditioning to investigate possible treatment strategies in PTSD.

2.3.5.2.1 Fear conditioning in animal models of PTSD

In PTSD, the traumatic event (unconditioned stimulus, US) gives rise to an unconditioned response (UR), which is characterised by emotional arousal, intense fear and autonomic responses. This UR is associated with cues such as sounds, smells, sights, etc (conditioned stimuli, CS) that were present during the occurrence of the original traumatic event. Because of this association of the CS with the US, the CS gives rise to conditioned responses (CRs) that can present even in the absence of the US. Fear conditioning therefore models this learned fear paradigm. It should be emphasised though, that when fear conditioning is used to investigate PTSD, it should not be regarded as an animal model of PTSD. Rather, it is only used to model certain behavioural responses of PTSD in order to investigate the neurobiology and neuropathology underlying these maladaptive behavioural responses. Translational animal models of PTSD should accurately simulate the complete pathological human condition within a controlled environment (Harvey 2011, Yehuda, Antelman 1993). These animal models of PTSD submit animals to various stressors to eventually induce a bio-behavioural syndrome in the animal that closely resembles the symptoms of the human syndrome (termed face validity), its neuropathology (termed construct validity) as well as its response to treatment (termed predictive validity)(see Section 2.3.6. and Harvey and Shahid 2011). In order to incorporate the above-mentioned attributes, animal models of PTSD have utilised acute intense stressors, e.g. electric shock, underwater trauma, and exposure of animals to a predator, aversive challenges and situational reminders of a life-threatening event (stress-restress) to more closely model the long-term effects on behavioural, autonomic and hormonal responses seen in humans with PTSD (Oosthuizen et al. 2005, Uys et al. 2006). One example of a validated animal model of PTSD is Single Prolonged Stress (SPS) (Liberzon et al. 1997, Yamamoto et al. 2009), in which rats are subjected to a 2-hour restraint stressor, followed by 20 min forced swimming and underwater trauma, followed by ether exposure until loss of consciousness.

Time-dependent Sensitization (TDS) (Harvey et al. 2003), is an expansion of this SPS paradigm, with a reminder of the trauma seven days later. Inescapable Shock-Learned Helplessness (IS-LH)(Bonne et al. 2004) is a paradigm in which animals are repeatedly exposed to a series of inescapable footshocks. The predator scent stress (PSS) animal model of PTSD subjects rats to cat odour for a predetermined amount of time, inducing intense stress in the animals (Kats, Dill 1998, Stam 2007a, Stam 2007b). In these animal models of PTSD, fear conditioning is then applied to investigate the neural mechanisms underlying the disorder in a pathologically equivalent model. In SPS, for example, after the application of the three consecutive stressors, the rodents are left undisturbed in their cages for a minimum of seven days to allow for pathological changes to take their course. Hereafter, behavioural assessments, such as fear conditioning, is conducted (Kohda et al. 2007, Madden IV et al. 1971, Brand et al. 2008), with or without various pharmacological interventions (Olson et al. 2011) and/or neurochemical studies (Knox et al. 2010) to determine the pathology underlying the specific maladaptive behavioural response observed in the human disorder.

2.3.6 VALIDITY OF ANIMAL MODELS OF FEAR CONDITIONING

Animal models provide us with a useful tool with which to investigate the neurobiology and treatment of a neuropsychiatric illness with behavioural implications, such as PTSD. They offer the possibility of simulating a condition under controlled circumstances that will enable us to study symptoms as they develop and to test prospective treatments (Yehuda, Antelman 1993). F. Josef van der Staay aptly defines the parameters required for a valid animal model:
"*An animal model with biological and/or clinical relevance in the behavioral neurosciences is a living organism used to study brain–behavior relations under controlled conditions, with the final goal to gain insight into, and to enable predictions about, these relations in humans and/or a species other than the one studied, or in the same species under conditions different from those under which the study was performed*" (van der Staay 2006).

Fear conditioning is an animal model of aversive learning and memory. Applying fear conditioning to an animal model of PTSD, such as SPS and TDS

described in section 2.3.5.2.1, not only aids in investigating the neural mechanisms underlying the disorder, but will also be helpful in testing efficacy of pharmacological agents in the treatment of the re-experiencing, hyperarousal and avoidance symptoms observed in PTSD. In order to apply this behavioural model in a translational animal model of PTSD, it should be validated as a veritable model in which PTSD-induced maladaptive responses can be assessed. The validity of the animal model determines the value and applicability of findings derived from the model to the human disorder. The validation of an animal model is a scientific method which improves the confidence in the interpretation of the data arising from this model (Holmes 2003). An animal model cannot be valid in all situations and for all purposes, and validity is restricted to a specific use for the model, and hence a model should always be open for re-evaluation (Silva 1993). Behavioural animal models are usually validated according to three criteria of validity, namely face, predictive and construct validity (Willner 1991).

2.3.6.1 Face validity

Face validity refers to the similarity of symptoms or the descriptive similarity of the behavioural dysfunction in the animal model vs. the human disorder (van der Staay et al. 2009). Therefore, an animal model of fear learning and memory should induce a significant learning of the association between the US and the CS in the animal, and this should be accompanied by an attempt to avoid the CS, as well as autonomic responses as seen in the stress response.

2.3.6.2 Predictive validity

Predictive validity, as used in psychopharmacology, is of particular importance in drug development programmes. Within this context, predictive validity refers to the ability of established pharmacological treatments to show comparable efficacy in the animal model (Swerdlow, Sutherland 2005). In an animal model of learning and memory, such as fear conditioning, predictive validity can be shown by using drugs known to abolish memory aquisition or consolidation, such as protein synthesis inhibitors infused directly into brain areas responsible

for memory consolidation (Cohen et al. 2006, Desgranges et al. 2008), muscarinic receptor antagonists (Grette Lydon, Nakajima 1992a) or noradrenergic receptor antagonists such as propranolol (Ferry et al. 1999a, Lennartz et al. 1996, Pitman et al. 2002).

2.3.6.3 Construct validity

Construct validity evaluates the similarity between the mechanisms underlying behaviour in the model and the mechanisms thought to underlie the human condition (van der Staay et al. 2009). Thus, the molecular and biological changes seen in the animal model should correlate with the pathophysiology of the human disorder. Van der Staay and colleagues argue that construct validity is the most important criterion for animal models because it addresses the soundness of the theories that underlie the model. Therefore, if the exact underpinnings of a human neuropsychiatric disorder are still under scrutiny or speculation, this criterion has evident shortcomings. For the present study, an animal model of fear conditioning should at least show enhanced glutamatergic and noradrenergic signaling, with increased levels of glucocorticoids and noradrenaline released in response to the training trials. Such a basis of validity will provide a solid base of evidence in support of the known mechanisms involved in the acquisition and consolidation of fear memory (De Kloet et al. 1999, Ferry et al. 1999a, Ferry et al. 1999b, Ferry, McGaugh 1999, Liang et al. 1986, Liang et al. 1990).

2.3.7 Selection of animals: Separation of the affected and behavioural cut-off criteria

PTSD affects between 15-25% of trauma-exposed individuals (Cohen et al. 2005, Yehuda 2009) and only a portion of exposed subjects show enhanced stress sensitivity. Thus, in order for an animal model to be regarded as closely modeling the human condition, it should be as valid as possible an approximation of the disorder as observed in humans. The concept of “setting apart the affected” has recently been studied in animal models of PTSD (Cohen et al. 2003, Cohen et al. 2004, Cohen et al. 2005). The authors question the validity of taking whole data of an entire population of animals exposed to a

stress paradigm, regardless of diverse range of responses, and interpreting the data of these entire populations as if it were a homogenous whole. They showed that in practice there is a considerable degree of variability among the animals with respect to the degree of behavioral disruption induced by the stressor (Cohen et al. 2003). The authors propose that since some animals appear to be more vulnerable to stress than others, these stress-sensitive or "behaviorally affected animals/maladaptive animals" should be set apart and used exclusively to study the pathology of stress-related disorders such as PTSD. This is not far removed from the stringent criteria for inclusion of human populations in studies investigating PTSD (American Psychiatric Association 2000). The authors propose the use of Behavioural Cut-off Criteria (CBC) to screen the animals in order to select the more clearly affected by stress, as is found in human PTSD. This application of CBC in an animal model of fear conditioning will more closely approximate that of the human condition. The selection of animals has been performed numerously in inhibitory avoidance (passive avoidance) paradigms, with studies using a training cut-off time as inclusion criteria for behavioural assessments following passive avoidance training (Angelucci et al. 1999, Browman et al. 2005, Piri, Zarrindast 2011). Based on such criteria, animals that exceed a predetermined latency to enter the dark chamber during the training session on day 1 of passive avoidance testing are removed from further experiments (see section 2.3.2.1).

In addition to applying CBC to provide a valid sample of stress sensitive animals, this study will also make use of Wistar rats because of their ability to show stronger conditioned and unconditioned responses to stress (Staples, McGregor 2006). By applying CBC, animals that exceed a latency of 60 s (Browman et al. 2005) to enter the dark compartment in the training trial of passive avoidance testing (see section 2.3.2.1) will be excluded from the study.

2.3.8 CONCLUSION

PTSD presents with symptoms of intrusive traumatic memories, re-experiencing of the trauma in the form of nightmares and flashbacks, persistant hyperarousal and inappropriate fearful responses to stimuli that are not (or no

longer are) threatening (American Psychiatric Association 2000). These memory intrusions can be very distressing to the patient and can aggravate the progression of the disorder in a snow-ball manner. Re-experiencing of the symptoms can cause reconsolidation of the traumatic memories, which in turn results in more vivid flashbacks and reliving of the trauma. These invasive, vivid relivings of the initial trauma can have a devastating effect on the patient's overall ability to cope and is extremely detrimental to the patient's social functioning (Kessler 2000, Yehuda 2002), physical health and emotional well-being (Woods, Wineman 2004).

Acquiring fearful memories is an indisposable adaptive response; however, responding to these memories in inappropriate contexts can be harmful. PTSD is appropriately considered to be a syndrome of maladaptive fear responses to a traumatic stressor (Olff et al. 2009). Learning and memory processes such as fear conditioning are thought to be the underpinnings of the maladaptive symptoms characteristic of this syndrome (Michael et al. 2005, Pitman 1989, Rothbaum, Davis 2003). Therefore, fear conditioning is a valuable tool in elucidating the mechanisms that fail to "shut down" and appropriately regulate the fear response after the trauma. Furthermore, it is also very valuable in investigating possible strategies for preventing and treating PTSD posttrauma.

2.4 PROJECT AIMS AND OBJECTIVES

2.4.1 PROJECT AIMS

This project set about to re-examine the diverse and complex role of noradrenergic receptors in the conditioned fear response, in this instance considering non-selective as well as selective β-adrenergic and α-adrenergic receptor agonists and antagonists and their response in a single fear conditioning model using an animal known to be stress-sensitive.

The aims of this study were the following:

- To set up and validate a passive avoidance fear conditioning paradigm in rats for application in future PTSD-related studies in our laboratory.
- To ascertain the efficacy of specific noradrenergic receptor modulators to attenuate or bolster fear conditioning in a passive avoidance paradigm in rats.

Ultimately, knowledge obtained from this study will be applied to future exploratory studies in a translational animal model of PTSD, such as SPS and/or TDS, in order to investigate whether the tested agents described above may exert similar effects on conditioned fear responses in a PTSD-like paradigm. These studies will have far-reaching implications for the treatment of PTSD, especially as a preventative strategy against the progression from acute stress syndrome immediately posttrauma to full-blown PTSD.

2.4.2 PROJECT OBJECTIVES

The objectives of the present study were:

- To validate the Gemini™ Avoidance System for use in our laboratory with regards to passive conditioned fear avoidance.

- To confirm the importance of a central mechanism of action for β-adrenoceptor blockade in the possible application of β-adrenoceptor blocking drugs in preventing the development of PTSD posttrauma.
- To investigate the role of α_1-, α_2-, β_1-, and β_2- receptors in a non-pathological classical conditioned fear avoidance paradigm in order to demonstrate how selective pharmacological modulation of these receptors may modify the fear response, and whether any of these receptor systems may exert opposing effects on passive fear conditioning.

This work will shed new light on the complex neurobiology of the fear response, and will have particular importance for our understanding of the neurobiology of PTSD and how its progression from acute stress syndrome to PTSD can be abrogated by selected pharmacological intervention.

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CHAPTER 3

Article

An investigation into the effect of various noradrenergic receptor modulators on fear memory consolidation in a passive avoidance paradigm

Article for submission for publication in *Psychopharmacology*

3.1 INTRODUCTION

This chapter presents the full-length manuscript for submission to *Psychopharmacology,* published by Springer. The manuscript is presented in the required format prescribed by the "Authors Instructions", which can be viewed on the journal website:
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The complete "Authors' Instructions" have been reproduced and is provided in Addendum B of this dissertation**.** As per "Authors Instructions" this manuscript consists of a title page including the title and contributing authors with their affiliations, addresses and contact details, followed on a separate page by the abstract of no more than 250 words, divided into subheadings as prescribed, and the keywords. Abbreviations are to be defined upon first mention in the abstract and in the text. The main body of the article follows on a new page in the prescribed format, with figures and legends to figures included in the text. References and citations are done in the format prescribed in the Authors' Instructions.

An investigation into the effect of various noradrenergic receptor modulators on fear memory consolidation in a passive avoidance paradigm

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ABSTRACT

Rationale Posttraumatic stress disorder (PTSD) is characterised by maladapative learned fear responses. Although the noradrenergic system plays a prominent role in fear conditioning, the role of individual noradrenergic receptors is less clear.

Objective In order to assess the role of separate adrenoceptors of relevance to effects on learned fear in PTSD, this study evaluated the effects of post-training administration of the β_1-antagonist betaxolol, β_2-antagonist ICI 118551, α_1-antagonist prazosin, α_2-agonist guanfacine and the α_2-antagonist yohimbine, and the non-selective β-antagonist, propranolol, in a contextual fear conditioning paradigm in rats.

Methods Male Wistar rats were subjected to a single trial passive avoidance paradigm where step-through latency to cross from a light to a dark chamber (where a footshock is administered) was recorded over two days. Immediately post-training, animals received subcutaneous injections of drug or vehicle (saline) control. Retention of the aversive memory for the dark context was assessed 24 hours later and latency to cross to the dark chamber was taken as an index of aversive learning. Propranolol was used as reference. Drugs were employed in a three-tiered dose response design to establish optimal dosages to modulate fear conditioning.

Results Betaxolol and ICI 118551 both showed equal and significant attenuation of contextual conditioned fear at 10mg/kg and 1mg/kg, respectively, and comparable to propranolol. Prazosin significantly diminished fear conditioning at 0.1mg/kg comparable to propranolol, while guanfacine and yohimbine showed no inhibitory effect.

Conclusions β_1 and β_2 receptors are equally important in conditioned fear responses, while α_1 inhibition (but not α_2 modulation) also mediates a pronounced inhibitory effect on learned fear responses. Selectively targeting α_1-, β_1- and β_2-noradrenergic receptors immediately posttrauma might prove useful as strategies to prevent the development of PTSD.

KEYWORDS: PTSD, Fear conditioning, Passive Avoidance, Learning and memory, Noradrenergic receptors, Wistar rat, propranolol

1. INTRODUCTION

Posttraumatic stress disorder (PTSD) is a severely debilitating anxiety disorder that can develop in the aftermath of a traumatic or life-threatening event in which extreme horror, intense fear or bodily harm has been incurred. The disorder presents with a symptom triad of re-experiencing (e.g. flashbacks, nightmares, intrusive thoughts), hyperarousal (e.g. hypervigilance, exaggerated startle response, sleep disturbances) and avoidance symptoms (avoiding places, events or people reminiscent of the trauma, emotional numbing)(American Psychiatric Association 2000). Apart from this symptom triad, PTSD is also characterised by cognitive impairments, including impaired attention and working memory function (Vasterling et al. 1998, Weber et al. 2005), impaired explicit memory (such as short-term memory loss) (Rauch et al. 2006, Shin et al. 2004) and impediments in new learning and memory acquisition (Golier et al. 2002). Failure to recover and adapt from responses to stress as well as dysfunctional extinction of fearful memories and maladaptive responses to these strong fearful memories is central to the symptom progression of PTSD (Charney 2004, Mahan, Ressler 2011).

Dysfunctional activity of multiple neurotransmitter pathways is implicated in the pathology underlying PTSD symptomotology, including noradrenergic, serotonergic, dopaminergic and glutamatergic systems, as well as the HPA axis (Friedman et al. 1994, Medina 2008, Ravindran, Stein 2009, Steckler, Risbrough 2011). These systems are also reciprocally interlinked, with dysfunction of one system affecting the function of the other. This significantly complicates any endeavour to understand the pathological mechanisms underlying PTSD, as well as how best to treat it. Further in-depth investigation is thus needed in order to delineate the causal role of each system, and thus to propose new avenues of treatment.

The noradrenergic system, in conjunction with cortisol, plays a vital role in the stress response and in the learning and memory of fear. The noradrenergic system also plays a prominent role in activating the HPA axis and modulating

the role of glucocorticoids in the stress response (Roozendaal et al. 2006b). Noradrenergic modulation in the basolateral amygdala (BLA) is furthermore vital in both glucocorticoid (Quirarte et al. 1997, Roozendaal et al. 1999, Roozendaal et al. 2002, Roozendaal et al. 2006a) and glutamatergic (Huang et al. 1994, Lennartz et al. 1996, Wang et al. 1996) mechanisms underlying memory consolidation. Maladaptive processing of emotional and trauma-related memories are thought to be causal in the development of PTSD (Debiec, LeDoux 2006), so that investigating the role of noradrenergic modulation of conditioned fear responses in PTSD becomes a vital means to understanding the underpinnings of this disorder as well as in designing appropriate pharmacotherapy.

Compared to neutral events, experiences of emotional or traumatic events form very strong memories (McGaugh 2002). These experiences are associated with increased noradrenergic levels in areas of the brain involved in learning and memory (McGaugh et al. 2002, McGaugh 2004, McIntyre et al. 2002). Emotionally aroused noradrenergic activation of the BLA has been shown to strengthen memory consolidation in other brain regions such as the hippocampus (McGaugh 2004) which is imperative for the formation of fear memories related to the context of the fearful event (Anagnostaras et al. 1999, Kim, Fanselow 1992, Maren et al. 1997). Together with changes in the amygdala and hippocampus, impaired inhibitory tone of the PFC on the fearful responses of the aforementioned regions is also an important component of the neurobiology of the disorder (Shin et al. 2004, Shin et al. 2006). Such knowledge has laid the foundation for the use of noradrenergic modulators in the treatment of PTSD, particularly the use of β-adrenergic blockers, such as propranolol, as treatment strategy to prevent the development of PTSD immediately posttrauma (Peskind et al. 2003, Pitman et al. 2002, Reist et al. 2001, Taylor et al. 2008b, Vaiva et al. 2003). The α_1-adrenergic blocker, prazosin, has also been used to treat nightmares associated with the disorder (Peskind et al. 2003, Raskind et al. 2003, Taylor et al. 2008a). However although certain studies have shown a role for α_1-antagonism in impairing emotional memory consolidation (Ferry et al. 1999b), others have shown

conflicting results (Lazzaro et al. 2010). There is also a great deal of controversy regarding the exact role of the various noradrenergic receptors in the molecular processes governing the acquisition, consolidation and expression of fear memory. In fact, it remains a moot point as to whether β_1 or β_2 adrenoceptors are the primary molecular substrate for propranolol's efficacy as a preventative strategy in the conversion of acute traumatic stress syndrome to full blown PTSD (Shalev et al. 2011).

A study by Qu et al (2008), demonstrated the facilitatory role of both β_1 and β_2-receptors in the BLA in consolidation of auditory fear conditioning in rats (Qu et al. 2008). However, it should be emphasized that auditory fear conditioning and contextual fear conditioning do not involve the same pathways in the brain. Moreover, the hippocampus also plays a critical part in the consolidation of contextual fear memories (Diehl et al. 2007, Harooni et al. 2009) while forming an integral part of PTSD symptomatology and possibly etiology (Acheson et al. 2011). Poor hippocampal-PFC signaling could underlie contextual memory deficits and conditioned fear responses lacking context sensitivity in PTSD (Acheson et al. 2011), and separate roles have been described for β_1- and β_2-receptors in memory processes involving the PFC. In this regard, non-selective β_2-receptor antagonism with propranolol has shown no effect on PFC function in monkeys, whereas the selective β_1-antagonist, betaxolol, was shown to enhance PFC function (Ramos et al. 2005), while a later study suggested that β_2-antagonism impaired PFC activity (Ramos et al. 2008). Understanding the distinct roles of selective noradrenergic receptors in fear memory consolidation remains an important field of interest, but is complicated by conflicting and opposing findings in the literature, thus making further investigation imperative.

The passive avoidance paradigm is a form of contextual learning employing a footshock as an aversive stimulus. Footshocks in rodents have been shown to induce the release of noradrenaline in the amygdala in varying quantities, and is directly correlated to the intensity of the shock (Galvez et al. 1996, Quirarte et al. 1998) and to the measure of memory retention for the event. (McIntyre et al. 2002).

In the present study, the selective roles of noradrenergic α- and β-receptors on consolidation of aversive memories were investigated in a step-through passive avoidance paradigm of fear conditioning employing a selected group of centrally acting noradrenergic drugs. The selective roles of the β_1- and β_2-receptors were investigated with the centrally acting β_1-selective antagonist betaxolol and the β_2-selective antagonist ICI 118551, employing the non-selective β-antagonist propranolol as a positive control. Selective α_1- and α_2-adrenergic responses were investigated using the centrally acting α_1-antagonist prazosin, α_2-agonist guanfacine and the α_2-antagonist yohimbine. Although yohimbine has been shown to aggravate symptoms of PTSD (Southwick et al. 1999) and to enhance retention of fear memory (Ferry et al. 1999c), it was employed in this study as the pharmacological antithesis to guanfacine in order to ascertain the specific role of the α_2-receptor on the consolidation of contextual aversive memories. In this study, we hypothesize that the non-selective β-blocker, propranolol, as well as the β_1-selective blocker, betaxolol, the β_2-selective blocker, ICI 118551 and the selective α_1-antagonist, prazosin, will attenuate the consolidation of contextual conditioned fear, while the α_2-agonist, guanfacine, will attenuate the consolidation of contextual conditioned fear in the passive avoidance paradigm. However, we propose that yohimbine will bolster the retention of conditioned fear.

2. MATERIALS AND METHODS

2.1 Animals

Male Wistar rats, weighing 180-230g, were selected for this study since these animals have been described as showing more robust conditioned and unconditioned responses to stress (Staples, McGregor 2006), thus improving the overall sensitivity and validity of the behavioural tests. Animals were housed in groups of five in standard laboratory cages (380 mm x 380 mm x 320 mm) at the Animal Research Centre of the North-West University under well-maintained environmental conditions, including temperature (21 ℃), and relative humidity (50 ± 5%). A positive air pressure with air filtration 99.7%

effective for a particle size of 2 micron and 99.9% for a particle size of 5 micron, as well as a 12 hour light/dark cycle was maintained, with lights off at 18h00. Animals were handled according to the guidelines stipulated by the Ethics Committee of the North West University (Ethics Approval Number: NWU-00007-11-S5).

2.2 Drugs

Propranolol hydrochloride, prazosin hydrochloride, yohimbine hydrochloride and guanfacine hydrochloride were purchased from Sigma Aldrich (Johannesburg, South Africa) and dissolved in sterile water for injection adjusted for the desired concentration and injected at a volume of 1ml/kg. All drugs were injected subcutaneously immediately post-training at the following doses: propranolol 10 mg/kg (Do-Monte et al. 2010, Rodriguez-Romaguera et al. 2009), betaxolol 1; 5 and 10 mg/kg (Murchison et al. 2004, Rudoy, Van Bockstaele 2007, Stone et al. 1996), ICI118551 0.4; 1 and 4 mg/kg (Murchison et al. 2004, Stone et al. 1996), prazosin 0.1; 1 and 5 mg/kg (Murchison et al. 2004, Stone et al. 1996), guanfacine 0.1; 0.3 and 1 mg/kg (Lê et al. 2011, Sagvolden 2006) and yohimbine 1; 5 and 10 mg/kg (Cain 2004, Holmes, Quirk 2010). The dose ranges were appropriately selected from the literature, as indicated. Furthermore, the overall study design and dose for propranolol (10 mg/kg) was also confirmed in a dose response pilot study conducted in our laboratory using the same paradigm (See Addendum A).

2.3 Apparatus and behavioural assessment

Passive Avoidance Fear Conditioning

Passive avoidance sessions were conducted using the computer-assisted GEMINI™ Avoidance System (San Diego Instruments, San Diego, CA, USA). The GEMINI™ consists of two equal chambers separated by a computer automated guillotine gate. The walls of each chamber are made of black Plexiglas and the floor consists of stainless steel rods facilitating the

administration of footshocks to the animal. Each chamber has 8 photobeams that detect the location of the animal so that a computer-assisted protocol can be employed. A one trial step-through procedure was used to measure the effect of the various drugs on the consolidation of contextual conditioned fear acquired after a single shock experience. The procedure was based on previously described studies employing the same passive avoidance paradigm (Díaz-Trujillo et al. 2009, Khakpour-Taleghani et al. 2008, Nielson et al. 1999, Piri, Zarrindast 2011). During the training trial on day one of behavioural testing, the animal was placed in one of the compartments with the gate closed and allowed to freely explore the context (compartment). After 30 seconds, this compartment was illuminated and the gate opened. When the animal crossed over to the dark compartment, the step-through latency (time in seconds) was recorded and the gate closed, whereafter a mild footshock of 0.8 mA was applied for 5 seconds. The shock intensity was based on earlier studies (Acosta et al. 2000, Bainbridge et al. 2008, Jafari-Sabet 2006), and corroborated by in-house pilot studies (see Addendum A).

Cut-off behavioural criteria were applied (Cohen et al. 2003) in order to have a more stress responsive population of animals in the study. Animals exceeding a step-through latency of 60 seconds on day 1 were removed from the study and replaced with a naive animal until a minimum of 10 animals per treatment group was attained (Browman et al. 2005). The animal was then left in the dark chamber for an additional 10 seconds to allow for contextual association (dark compartment) with the footshock. Upon removal of the animal from the apparatus, it immediately received a subcutaneous injection of test drug, with control animals receiving an equivalent volume of saline. Twenty-four hours later, retention of the aversive memory was tested. The procedure was now repeated with the exception that no shock was administered upon entrance into the dark compartment.

2.4 Experimental design

Animals were randomly divided into drug and saline treatment cohorts, with a minimum of 10 animals per treatment group. Behavioural assessments were performed over two days: on the first day, a single passive avoidance training trial was conducted. After passive avoidance training, each animal received the relevant subcutaneous drug administration and were returned to their home cages. Twenty-four hours later, the passive avoidance retention trial was conducted with a cut-off time of 600 seconds. No drugs were administered after the retention trial on day two.

2.5 Statistical analysis

In accordance with earlier work (Ferry et al. 1999b, Ferry, McGaugh 1999), only the step-through latencies as measured on day 2 are used for statistical analysis. Propranolol and saline were used as active and placebo comparator, respectively, for the β-adrenergic studies, with saline used as placebo comparator for the α-adrenergic studies. Comparisons among mean retention latencies for the different treatment groups were performed with one-way analyses of variances (ANOVA) to determine if statistically significant differences existed between the means of the step-through latencies. Levene's tests were performed to assure equality of variances in each ANOVA's case. In the case of unequality of variances, Welch tests were performed, since the assumption for normal ANOVA was not met. Normal probability plots on the residuals were done to assure that the data was fairly normally distributed (Tabachnick, Fidell 2001). Post hoc Dunnett's tests were done to determine differences in the means between test groups and saline controls or between test groups and propranolol. Based on data described in an earlier pilot study (see Addendum A), locomotor determination was not deemed necessary. These procedures were performed using the statistical data analysis software system STATISTICA (StatSoft, Inc. 2007) version 8.0. All tests were done at a 0.05 significance level.

3. RESULTS

Training latencies on day 1 for all treatment cohorts before administration of saline or adrenergic drugs were below 60 s due to the 60 s cut-off time and the mean ± SEM training latencies are shown in Table 1. Exact latency and p-values for individual comparitor studies as indicated are summarised in Table 2-3 below each graph. The step-through retention latencies of rats treated with β-adrenergic blockers are shown in Fig. 1 and results summarised in Table 2. ANOVA indicated significant differences in step-through latencies between test versus control groups in the retention test with a Welch p-value of 0.0001. The mean retention latency of the saline control group was 453.18 ± 76.77 s, compared to training latency of 28.91 ± 4.24 s, indicating that footshock induced a significant retention of inhibitory avoidance training. Dunnett's post hoc test revealed that post-training administration of the non-selective β-antagonist, propranolol, at a dose of 10 mg/kg, produced a significant deficit in retention of the aversive memory compared to saline controls with mean ± SEM retention latency of 90.93 ± 58.66 s and a p-value of 0.005. Post-training administration of only the highest dose of the β_1-antagonist betaxolol, i.e. 10 mg/kg, also induced a significant impairment in retention latency (102.99 ± 60.19 s) versus saline control with a p-value of 0.008. The β_2-antagonist ICI 118551 induced a comparable impairment in memory consolidation at a dose of 1 mg/kg, with a mean retention latency of 101.71 ± 41.62 s versus saline control and p=0.007. Post-training administration of the 1 mg/kg and 5 mg/kg doses of betaxolol and the 0.4 mg/kg and 4 mg/kg dose of ICI 118551, did not significantly impair retention latencies compared to the saline control group.

Table 1 Mean training latencies in a step-through passive avoidance task for all treatment cohorts prior to treatment with either saline (control), or the β_1/ β_2 receptor antagonist propranolol 10 mg/kg (Prop 10mg)(reference drug), the β_1-selective antagonist betaxolol (Betax 1; 5 and 10 mg/kg), the β_2-selective antagonist ICI 118551 (ICI 0.4; 1 and 4 mg/kg), the α_1-selective antagonist prazosin (0.1; 1 or 5 mg/kg), the α_2-selective agonist guanfacine (0.1; 0.3 or 1 mg/kg), or the α_2-selective antagonist yohimbine (1, 5 or 10 mg/kg). These data was not used for statistical analyses, as stated in the text. n=10 for each group

TREATMENT COHORT	Mean Training Latency (s) (Mean ± SEM)
Saline	28.91 ± 4.24
Propranolol 10 mg/kg	22.35 ± 3.55
Betaxolol 1 mg/kg	19.37 ± 4.28
Betaxolol 5 mg/kg	16.6 ± 3.60
Betaxolol 10 mg/kg	14.86 ± 2.97
ICI 118551 0.4 mg/kg	15.14 ± 2.86
ICI 118551 1 mg/kg	11.25 ± 1.90
ICI 118551 4 mg/kg	17.27 ± 3.85
Prazosin 0.1 mg/kg	15.99 ± 2.81
Prazosin 1 mg/kg	19.63 ± 2.93
Prazosin 5 mg/kg	13.47 ± 2.83
Guanfacine 0.1 mg/kg	13.57 ± 2.05
Guanfacine 0.3 mg/kg	20.93 ± 5.07
Guanfacine 1 mg/kg	16.97 ± 2.41
Yohimbine 1 mg/kg	12.17 ± 1.99
Yohimbine 5 mg/kg	20.77 ± 2.55
Yohimbine 10 mg/kg	15.17 ± 3.10

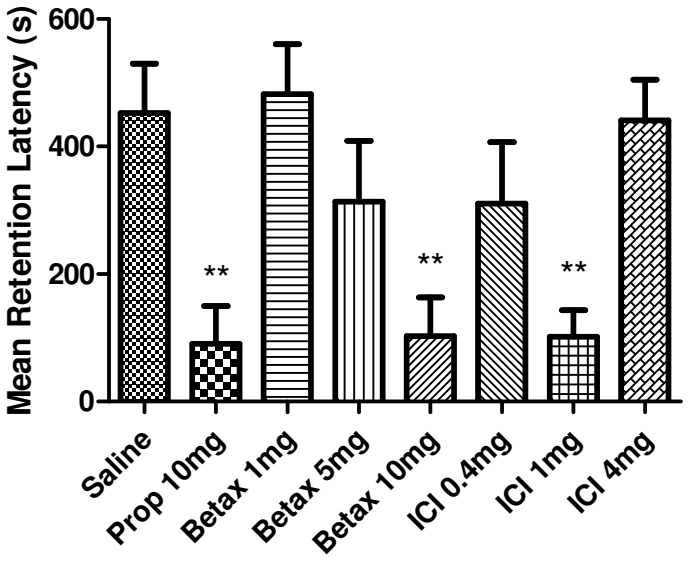

Fig. 1 Mean retention latencies (mean ± SEM; s) in a step-through passive avoidance task in rats treated with either saline (control), or the β_1/ β_2 receptor antagonist propranolol 10 mg/kg (Prop 10mg)(reference drug), the β_1-selective antagonist betaxolol (Betax 1; 5 and 10 mg/kg) or the β_2-selective antagonist ICI 118551 (ICI 0.4; 1 and 4 mg/kg). n=10 for each group. *p<0.05, **p<0.01, compared to saline control group (ANOVA and Dunnett's post hoc)

Table 2 Effect of post-training administration of the central β_1/β_2 receptor antagonist propranolol (10 mg/kg), the β_1-selective antagonist betaxolol (1; 5 and 10 mg/kg) or the β_2-selective antagonist ICI 118551 (0.4; 1 and 4 mg/kg) on passive avoidance retention latencies. Values are expressed as mean ± SEM (s) of 10 animals per group. Dunnett's p-values for each drug treatment compared to saline is given.

DRUG TREATMENT	Mean Retention Latency (s) (Mean±SEM)	Dunnett's post hoc p-value (compared to saline control)
Saline	453.18 ± 76.77	
Propranolol 10 mg/kg	90.93 ± 58.61	**0.005**
Betaxolol 1 mg/kg	482.36 ± 78.42	0.999
Betaxolol 5 mg/kg	313.23 ± 95.63	0.623
Betaxolol 10 mg/kg	102.99 ±60.19	**0.008**
ICI 118551 0.4 mg/kg	310.18 ± 96.65	0.602
ICI 118551 1 mg/kg	101.71 ± 41.62	**0.007**
ICI 118551 4 mg/kg	441.46 ± 63.46	1.0

Step-through latencies of rats treated with the α-adrenergic drugs are shown in Figure 2 and Table 3. ANOVA indicated significant differences in step-through latencies between test and control groups in the retention test with a Welch p-value = 0.000006. Dunnett's post hoc test revealed that post-training administration of the α_1-antagonist prazosin significantly impaired retention compared to saline controls at a dose of 0.1mg/kg, with p-value = 0.008 and mean retention latency of 115.31 ± 61 s. The 1mg/kg dose of prazosin also impaired the retention latencies compared to saline, although this dose failed to reach statistical significance with p= 0.065 and a mean retention latency of 187.69 ± 78.16 s. The 5mg/kg dose of prazosin did not show any impairment of retention latency. The α_2-selective antagonist, guanfacine, did not impair retention in the passive avoidance task at any of the doses tested. However, the highest dose of 1mg/kg showed a slight attenuation of retention latency with a mean retention latency of 245.7 ± 80.41 s, thus lower than that of the saline controls but not reaching statistical significance (p= 0.231, n.s). The α_2-antagonist, yohimbine, tended to bolster the mean retention latencies of animals versus saline control, with 1, 5 and 10mg/kg doses inducing mean latencies of 542.61 ± 57.4 s, 548.2 ± 35.86 s and 555.85 ± 29.52 s respectively, all of which are very close to the cut off time of 600s. However, these effects did not reach statistical significance.

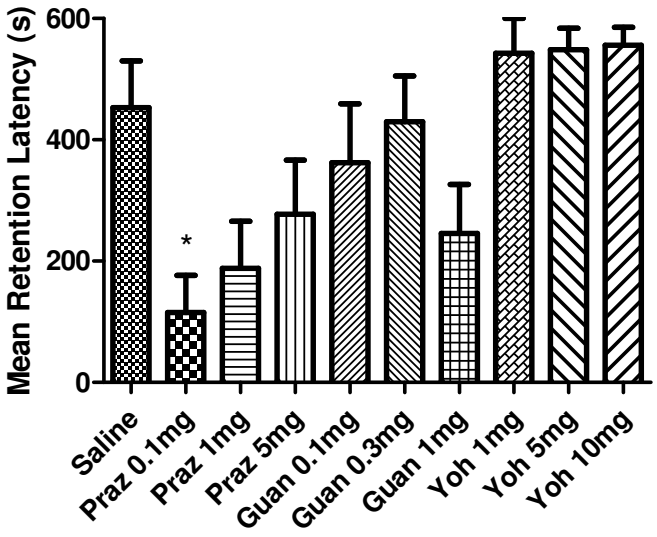

Fig 2 Retention latencies (mean ± SEM; sec) in a step-through passive avoidance task in rats treated with either saline (control), the α_1-selective antagonist prazosin (0.1; 1 or 5mg/kg), the α_2-selective agonist guanfacine (0.1; 0.3 or 1mg/kg) or the α_2-selective antagonist yohimbine (1, 5 or 10mg/kg). n=10 for each group. *$p<0.05$, compared to saline control group (ANOVA and Dunnett's post hoc)

Table 3 Effect of post-training administration of the α_1-selective antagonist prazosin (0.1; 1 or 5 mg/kg), the α_2-selective agonist guanfacine (0.1; 0.3 or 1 mg/kg) or the α_2-selective antagonist yohimbine (1, 5 or 10 mg/kg) on passive avoidance retention latencies. Values are expressed as mean ± SEM (s) of 10 animals per group. Dunnett's p-values for each drug treatment compared to saline is given.

DRUG TREATMENT	Mean Retention Latency (s) (Mean±SEM)	Dunnett's post hoc p-value (compared to saline control)
Saline	453.18 ± 76.77	
Prazosin 0.1 mg/kg	115.31 ± 61.02	**0.009**
Prazosin 1 mg/kg	187.69 ± 78.16	0.065
Prazosin 5 mg/kg	277.19 ±89.25	0.401
Guanfacine 0.1 mg/kg	362.22 ± 97.08	0.945
Guanfacine 0.3 mg/kg	429.59 ± 75.58	0.999
Guanfacine 1 mg/kg	245.7 ± 80.41	0.231
Yohimbine 1 mg/kg	542.61 ± 57.4	0.950
Yohimbine 5 mg/kg	548.2 ± 35.86	0.930
Yohimbine 10 mg/kg	555.85 ± 29.52	0.897

Figure 3 compares the dose of each noradrenergic active drug that induced the greatest change (i.e. improvement or deficit) in memory retention for the passive avoidance task, based on the mean retention latencies, and compared this to saline and to propranolol (reference comparator). ANOVA indicates a significant difference between retention latencies of the different drug treatment groups, with a Welch p-value of 0.000017. Dunnett's post hoc test reveals a significant difference between the retention latencies of the saline control group and the groups receiving post-training administration of propranolol 10 mg/kg (90.93 ± 58.61 s, p = 0.0008), betaxolol 10 mg/kg (102.99 ± 60.19 s, p = 0.0013), ICI118551 1mg/kg (101.71 ± 41.62 s, p = 0.0013) and prazosin 0.1 mg/kg (115.31 ± 61.02 s, p = 0.0020). When propranolol 10 mg/kg is used as a reference, Dunnett's post hoc test reveals a statistically significant difference between propranolol 10 mg/kg and saline and yohimbine 1 mg/kg treated

groups, with p = 0.0084 and p = 0.00003 respectively. However, all other test drugs were no different to that of propranolol, indicating equal efficacy. The p-values for the Dunnett's post hoc tests with saline and propranolol as reference groups are shown in table 4 and table 5 respectively.

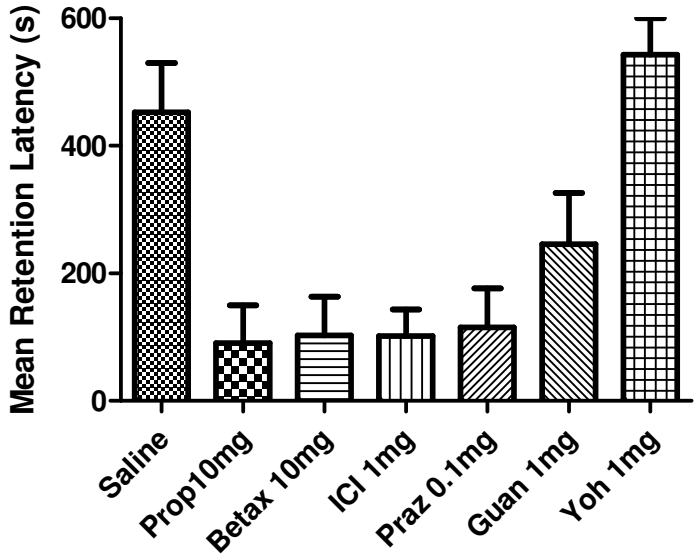

Fig 3 Comparative efficacy of the various noradrenergic drugs in attenuating contextual fear conditioning at their most effective dose. Data describes retention latencies (mean ± SEM; s) in a step-through passive avoidance task in rats treated with either saline (control), propranolol (10 mg/kg; reference), β_1-selective antagonist betaxolol (10 mg/kg), β_2-selective antagonist ICI 118551 (1 mg/kg), α_1-selective antagonist prazosin (0.1 mg/kg), α_2-selective agonist guanfacine (1 mg/kg) or the α_2-selective antagonist yohimbine (1 mg/kg). n=10 for each group. Statistical analysis versus saline and versus propranolol are described in tables 3 and 4, respectively.

Table 4 Comparative efficacy of the most effective doses of the various noradrenergic drugs in attenuating passive avoidance compared to saline controls. Dunnett's p-values for each drug treatment compared to saline is given.

DRUG TREATMENT	Dunnett's post hoc p-value (compared to saline control)
Saline	
Propranolol 10 mg/kg	**0.000847**
Betaxolol 10 mg/kg	**0.001310**
ICI 118551 1 mg.kg	**0.001251**
Prazosin 0.1 mg/kg	**0.002030**
Guanfacine 1 mg/kg	0.106704
Yohimbine 1 mg/kg	0.822552

Table 5 Comparative efficacy of the most effective doses of the various noradrenergic drugs in attenuating passive avoidance compared to propranolol 10 mg/kg. Dunnett's p-values for each drug treatment compared to propranolol 10 mg/kg is given.

DRUG TREATMENT	Dunnett's post hoc p-value (compared to propranolol)
Propranolol 10 mg/kg	
Saline	**0.000847**
Betaxolol 10 mg/kg	0.999996
ICI 118551 1 mg/kg	0.999998
Prazosin 0.1 mg/kg	0.999710
Guanfacine 1 mg/kg	0.333630
Yohimbine 1 mg/kg	**0.000033**

4. DISCUSSION

Memory processing is divided into distinct stages, namely acquisition, consolidation (and reconsolidation) and retrieval (Milner et al. 1998). *Memory acquisition* occurs when the animal learns to associate the context with an impending aversive footshock. An association between the context and the receiving of a footshock is thus acquired and this is stored as a labile memory. Memory consolidation is then required to transform this memory into a more fixed state, and requires prolonged noradrenergic activation (Barros et al. 2001, Gallagher et al. 1977, Ikegaya et al. 1997, Roozendaal et al. 1997). Finally, retrieval is the stage where the actual recollection of the memory of the context-foot shock-association takes place (Abel, Lattal 2001). In the current study, since the drugs were administered on day one immediately after memory acquisition, we have studied the effect of noradrenergic manipulation on the process of consolidation of the aversive memory following the initial acquisition of an aversive association with a context (on day 1 of testing), as measured by spontaneous recall of the adverse contexual association on day 2 (Roesler, Schröder 2011). This was done by measuring the retention of the aversive association of the context with the footshock on day 2 in the retention trial, expressed as strep-through latency to enter the dark compartment where the footshock was originally experiened. The extent to which the association between the footshock and the context is made and how it is consolidated into long term memory is measured by the animal's reluctancy to enter the dark chamber and by remaining in the light chamber. The measure of fear memory consolidation is therefore correlated with aversion to enter the dark chamber and is expressed as memory retention. No drugs were administered on day 2, and noradrenergic involvement specifically in modifying memory retrieval or extinction was therefore not assessed. In PTSD, this manner of testing is analogous to assessing flashbacks and memory intrusions that serve to reinforce the memory trace of the traumatic event.

The most important observations made in this study are that the non-selective β-receptor antagonist, propranolol, significantly inhibited memory consolidation for the fear-related passive avoidance task at a dose of 10 mg/kg (Figure 1; Table 1; Table 3) and that both the β_1-selective antagonist betaxolol (10 mg/kg) as well as the β_2-selective antagonist ICI 118551 (1 mg/kg) were as effective as propranolol in preventing fear memory consolidation and spontaneous recall (Figure 3, Table 4) The α_1-selective antagonist prazosin (0.1 mg/kg) was also found to be significantly effective versus saline control (Figure 2, Table 2; Table 3) and equal in efficacy compared to propranolol 10 mg/kg (Figure 3; Table 4). However, neither the α_2-selective agonist guanfacine (1 mg/kg) nor the α_2-selective antagonist yohimbine (1 mg/kg), were effective in this regard (Figures 2 and 3, Tables 2, 3 and 4). It would thus appear that the ability of propranolol to attenuate fear conditioned responses may be mediated by an action on both β_1- *and* β_2- receptors, while α_1-inhibition (but not α_2-modulation) also mediates a pronounced inhibitory effect on learned fear responses.

A number of studies have demonstrated the efficacy of β-blockers to inhibit memory consolidation of an emotional memory in animals (Barros et al. 2001, (Cahill et al. 1994, Gallagher et al. 1977, Ikegaya et al. 1997, Roozendaal et al. 1997, Roozendaal et al. 2004) and humans (Grillon et al. 2004, Harmer et al. 2001, Pitman et al. 2002). Less is however known on the effect of individual antagonism of the β_1- and β_2-receptors in contextual fear memory in an inhibitory fear conditioning paradigm. Opposite roles for β_1- and β_2-receptors have been proposed in other paradigms of stress and memory processing, and while propranolol's efficacy in preventing the development of PTSD is debated (Cohen et al. 2011, McGhee et al. 2009, Sharp et al. 2010, Stein et al. 2007), there might be a role for selective β_1- or β_2-antagonism in preventing the onset and development of PTSD. Poor hippocampal-PFC signaling could underlie contextual memory deficits in PTSD, which would make for poor contextual control of conditioned fear responses (Acheson et al. 2011). Where propranolol has shown no effect on PFC function in monkeys, β_1-selective antagonism with betaxolol was shown to enhance PFC function, which should improve PFC inhibitory control over the hippocampus and amygdala and consequently result

in greater control over traumatic memories (Ramos et al. 2005). A later study suggested that β_2-antagonism impaired PFC activity (Ramos et al. 2008), which would impair inhibitory tone on the amygdala and could subsequently enhance traumatic memory traces. Since the PFC exerts inhibitory control over the emotional processes of the amygdala (Shin et al. 2004), these findings would suggest that β_1-antagonism attenuates amygdala-mediated consolidation of fear memory, while β_2-antagonism should enhance this process. Furthermore, β_1-selective antagonism with betaxolol reverses the effect of stress on risk assessment behaviour in mice (Stone et al. 1996), an effect not shown with the β_2-selective antagonist ICI 118551. In another recent study, the β_2-antagonist ICI 118551 but not the β_1-antagonist betaxolol blocked forced swim stress-induced cocaine reinstatement (Vranjkovic et al. 2011). Schutsky et al (2011) in turn showed that memory retrieval is dependent on β_2- but not β_1-adrenergic receptors, concluding that the β_2-receptor could be a critical effector of acute stress, and that both β_1- and β_2-receptors have distinct roles in memory and cognition (Schutsky et al. 2011). However, in the present study we have showed that both β_1- and β_2-receptor antagonism attenuates fear memory consolidation in an inhibitory avoidance paradigm in a comparable and dose dependent manner. Indeed our data suggest that both receptors are equally adept at mediating the consolidation of fear memory, since suppression of fear conditioning engendered by these selective β-adrenergic antagonists was comparable to that induced by the non-selective β-antagonist, propranolol. These findings are similar to that described by Qu et al (2008) using an auditory fear conditioning paradigm.

The selective α_1-receptor antagonist, prazosin, significantly impaired aversive memory consolidation at a dose of 0.1 mg/kg. This is in line with studies showing the importance of the α_1-receptor in memory formation and consolidation processes (Ferry et al. 1999a, Ferry et al. 1999b). Furthermore, blockade of α_1-receptors has been shown to protect PFC function and weaken amygdala function in animals (Ferry et al. 1999d), which would result in a diminished fear response. Considering the important control that the PFC exerts on fear and explicit memory function, as determined by the amygala and

hippocampus respectively, this protective effect on PFC function by α_1-antagonism is a potentially viable tool in the prevention of the development of PTSD. High levels of noradrenaline are released during stress, and in the PFC α_1-mechanisms predominantly tone down higher cognitive processing, subsequently allowing basic survival mechanisms to govern behavioural responses (Birnbaum et al. 1999). Prazosin has also been used succesfully to reduce trauma-related nightmares in patients with PTSD (Peskind et al. 2003, Raskind et al. 2003, Thompson et al. 2008) and shows potential in treating some of the symptoms of the disorder. This study provides additional evidence for the role of α_1-mechanisms in contextual memory storage such as those involved in the inhibitory avoidance paradigm, and supports the clinical use of prazosin in PTSD, especially in secondary prevention of the disorder.

The selective α_2-agonist, guanfacine, did not significantly impair aversive memory consolidation. This is in contrast with studies showing efficacy of α_2-agonists in impairing memory and fear memory consolidation in animals (Davies et al. 2004, Ferry, McGaugh 2008, Gibbs et al. 2010). Presynaptic α_2-adrenoceptors are thought to exert a tonic inhibitory influence on noradrenaline release, particularly during stress, and the activation of α_2-receptors in the BLA has been shown to impede fear memory consolidation (Ferry, McGaugh 2008). The efficacy shown by prazosin to attenuate fear conditioning (Ferry et al. 1999b), as well as its efficacy in treating some of the symptoms of PTSD in humans (Raskind et al. 2003), leaves us with a challenge in interpreting the inefficacy of guanfacine to exert an inhibitory effect on fearful memory consolidation. By presynaptic agonism of the α_2-autoreceptor, guanfacine lowers the availability of synaptic noradrenaline for all adrenergic subtypes, whereas prazosin specifically blocks the postsynaptic α_1-receptor, exerting no effect on other adrenergic receptors. This universal lowering of synaptic noradrenaline by guanfacine might elicit a mixture of positive and negative influences on fear memory, which may abolish any beneficial effects (Neylan et al. 2006). In contrast and in line with its synaptic actions, the α_2-antagonist, yohimbine, tended to bolster the memory for the aversive event, although, we were unable to demonstrate a robust response under our experimental

conditions. Nevertheless, the ability of yohimbine to induce anxiety in humans (Charney et al. 1992, Southwick et al. 1993) and animals (Blanchard et al. 1993, Venault et al. 1993), to promote fear memory consolidation and to enhance associative fear (Soeter, Kindt 2011) and emotional memory consolidation when administered after stimulus presentation (Southwick et al. 2002), suggests that re-evaluation of these data, possibly with a greater number of animals, is warranted. More recently, targeting specific α_2-subreceptors has been suggested as an attractive future option to investigate in this paradigm of contextual fear conditioning, especially in conjunction with a translational animal model of PTSD (Krystal, Neumeister 2009). Franowicz and colleagues (2002) showed that a functional loss of the α_{2A}-adrenoceptor reduced performance in tasks requiring PFC cognitive processes. Considering the inhibitory role of the PFC in amygdalar and hippocampal fear responses, α_{2A}-receptor agonism should therefore theoretically produce potent inhibitory effects on aversive memory consolidation in the BLA, and therefore the α_2-receptor remains a target of interest in the treatment of memory-related symptoms of PTSD.

Except for betaxolol and prazosin, it was difficult to confirm the response of the drugs as being dose-dependent. Betaxolol showed a significant effect on fear conditioning that increased in step-wise fashion as a function of escalating dose. Thus, while lower doses were ineffective, the highest dose tested (10 mg/kg) was found to engender a significant attenuation of fear conditioning. Prazosin, on the other hand, *lost* efficacy as a function of escalating dose, with its most significant effect observed at the lowest dose tested (0.1mg/kg). ICI 118551, however, demonstrated a U-shaped response, with the drug exerting its most significant effect at the second highest (middle) dose (1 mg/kg), while both higher and lower dosages were ineffective. What is interesting is that this U-shaped dose-response was also produced by propranolol, suggesting that this unique dose reponse is probably mediated by β_2-receptor antagonism. The response to guanfacine or yohimbine, however, did not show any clear relation with dose. A dose-dependent effect of the latter two drugs on fear memory processing could therefore not be shown, and warrants further study to confirm

these observations. This might be due to individual effects of the drugs on the activity and state of consciousness of the animals, and effects of the drug on locomotor activity of unshocked animals might be helpful in the future to clarify these anomalies. Furthermore, the use of a wider dosage range as well as a larger number of animals might in future studies grant greater insight into these apparently confounding results.

In conclusion, despite the above-noted anomalies regarding the dose-dependent response of some compounds, this study has provided new insight into the selective roles of β_1- and β_2-receptor antagonism in contextual fear conditioning in rats, showing similar efficacy in attenuating conditioned fear. Similarly, selective α_1-antagonism is effective in attenuating conditioned fear, providing motivation for the efficacy of α_1-adrenergic antagonists in treating PTSD. Furthermore, the inefficacy of the α_2-selective agonist, guanfacine, to attenuate contextual fear conditioning warrants investigation into more selective α_2-receptor subtypes as potential targets for the treatment of PTSD. We have therefore confirmed the proposed hypothesis that non-selective $\beta_{1/2}$, as well as selective β_1-, β_2-, and α_1-antagonism impair retention of contextual conditioned fear. Furthermore we have shown that the efficacy of the latter three compounds is comparable to propranolol, a drug which is widely used to impair fear memory processes. Although not robust, we also confirmed the hypothesis that α_2-antagonism with yohimbine will bolster contextual conditioned fear retention. However, we failed to confirm the hypothesis that α_2-agonism with guanfacine will impair conditioned contextual fear consolidation. Since this work has focused on immediate post-training administration of these drugs, both α_1-, as well as β_1- and β_2-antagonists, may have application in the immediate aftermath of trauma to prevent the development of acute stress syndrome to full-blown PTSD.

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CHAPTER 4 Discussion & Conclusions

4.1 NORADRENERGIC RECEPTORS HAVE A DISTINCT ROLE IN FEAR CONDITIONING AND CONTEXTUAL FEAR MEMORY CONSOLIDATION

Posttraumatic stress disorder is a severely debilitating anxiety disorder that can develop in the aftermath of a traumatic or life-threatening event involving extreme horror, intense fear or bodily harm. The disorder presents with a symptom triad consisting of re-experiencing (e.g. flashbacks, nightmares, intrusive thoughts), hyperarousal (e.g. hypervigilance, exaggerated startle response, sleep disturbances) and avoidance symptoms (avoiding places, events or people reminiscent of the trauma, emotional numbing) (American Psychiatric Association 2000). Dysfunctional adaptive responses of multiple neurobiological pathways is implicated in the pathology underlying these symptom clusters, with the noradrenergic system playing a very prominent role (Geracioti et al. 2001, Morgan et al. 1993, Pitman et al. 2002, Roozendaal et al. 2004, Southwick et al. 1993). Through modulation of other pathways involved in the stress response, including glutamatergic (Huang et al. 1994, Lennartz et al. 1996, Wang et al. 1996) and glucocorticoid signaling (Quirarte et al. 1997, Roozendaal et al. 1999, Roozendaal et al. 2002, Roozendaal et al. 2006), the noradrenergic system is an important target in approaches focusing on preventing the development of PTSD in the aftermath of the trauma (Vaiva et al. 2003).

The re-experiencing cluster of symptoms is thought to be due to inadequate control over the fearful memory traces formed at the trauma, possibly related to increased HPA axis negative feedback and hypocortisolemia (Liberzon et al. 1999, Yehuda et al. 1991, Yehuda 2005, Yehuda 2009) and this is facilitated by

sustained hyperadrenergic activity in the aftermath of the trauma, resulting in very strong fearful memories which often lack context sensitivity. These explicit memories often give rise to intrusive thoughts, flashbacks and vivid nightmares (Debiec, LeDoux 2006) and also lead to avoidance symptoms and constant hyperarousal. It is thus of note that a number of clinical (Pitman et al. 2002, Vaiva et al. 2003) and pre-clinical (Adamec et al. 2007, Cohen et al. 2011b) studies have investigated using noradrenergic receptor antagonists, such as the non-selective $\beta_{1/2}$-antagonist, propranolol, as a secondary treatment strategy to prevent the development of PTSD immediately posttrauma (Zohar et al. 2011). However, there remains controversy as to the precise role of the different noradrenergic receptors in the process of fear memory consolidation and recall, and how this may determine symptom development on the one hand and response to secondary pharmacological intervention on the other. Moreover, although a number of such studies have been conducted in animals (Ferry et al. 1999a, Ferry et al. 1999b, Ferry, McGaugh 1999, Liang et al. 1986, Liang et al. 1990), many of these studies have used different fear conditioning paradigms and protocols, as well as different animals, which in the end complicates interpretation of the data and its possible validity for the human disorder.

In the present study, we employed a contextual fear conditioning paradigm to explore the effect of selective modulation of β_1-, β_2-, α_1-, and α_2-adrenoceptors on post-training consolidation of fearful memories in Wistar rats, a strain known to display increased fear responding in this protocol (Staples, McGregor 2006). The first objective of the study was to validate the Gemini™ Avoidance System for use in our laboratory using a passive avoidance paradigm, and to confirm that a known β-adrenergic receptor antagonist, propranolol, is capable of inhibiting fear conditioning congruent with data described in the literature, thereby validating the Gemini™ Avoidance System for use in our laboratory. Secondly, it would provide us with a meaningful effective dose that would be applied in the main treatment study where the effect of various α- and β-selective noradrenergic drugs would be tested with regard to their effects on this fear conditioning paradigm.

The non-selective β-receptor antagonist, propranolol, was therefore first tested over a wide dosage range in a passive avoidance paradigm to target consolidation of learned fear, and comparing the effect of propranolol to that of the peripherally acting β-antagonist, nadolol. Nadolol was selected not only to more convincingly demonstrate the efficacy of propranolol to inhibit fear memory consolidation, but also to demonstrate that these actions rest primarily on a central mode of action. The drugs were tested immediately post-training in order to study their respective role in fear memory consolidation. Propranolol's role in inhibiting the consolidation of emotional memories is well-studied (Barros et al. 2001, Cahill et al. 1994, Gallagher et al. 1977, Ikegaya et al. 1997, Roozendaal et al. 1997, Roozendaal et al. 2004), and the centrally-acting efficacy of propranolol in attenuating fear conditioning in rats would constitute a valuable form of predictive (pharmacological) and construct (mechanistic) validition of the contextual fear conditioning paradigm. Data presented in a pilot study (Addendum A) found that propranolol 10 mg/kg significantly inhibited the consolidation of learned fear in rats. This result and the effective dose described is in line with current literature on the role of centrally acting β-receptor blockade in learned fear paradigms in rodents, evident at this same (Nielson et al. 1999, Przybyslawski et al. 1999) as well as different doses of propranolol (Barros et al. 2001, Cahill et al. 1994, Gallagher et al. 1977, Ikegaya et al. 1997, Roozendaal et al. 1997, Roozendaal et al. 2004), and is also congruent with data in humans (Grillon et al. 2004, Harmer et al. 2001, Pitman et al. 2002). Interesting though, is recent findings that have reported that propranolol administered immediately post-training is ineffective in attenuating contextual fear conditioning in an animal model of PTSD (Cohen et al. 2011a). Nevertheless, this validation has confirmed the role of centrally acting β-receptor antagonism in attenuating fear conditioning in rats, and that propranolol 10 mg/kg is an effective dose for use as a reference drug in subsequent studies.

Based on the above findings, the second objective of this study was to examine the effect of targeting selective β_1-, β_2-, α_1-, and α_2-adrenoceptors in the same passive avoidance paradigm of contextual fear conditioning applied in the above pilot study. A dose response design with antagonists selectively targeting the β_1- and β_2-receptors, viz. betaxolol (at a dose of 10 mg/kg) and ICI 118551 (at a dose of 1 mg/kg) respectively, showed that both receptor subtypes are equally important in facilitating the consolidation of learned fear. Important was that the response to these two drugs was equal in efficacy to propranolol, the reference compound for this study. This is in line with a study by Qu et al (2008), who demonstrated the facilitatory role of both β_1- and β_2-receptors, using a posttraining drug administration protocol in the BLA in consolidation of auditory fear conditioning in rats (Qu et al. 2008). However, it should be emphasized that auditory fear conditioning and contextual fear conditioning do not involve the same pathways in the brain, with the hippocampus playing a more critical part in the consolidation of contextual fear memories (Diehl et al. 2007, Harooni et al. 2009), and also forming an integral part of PTSD symptomatology and possibly etiology (Acheson et al. 2011). Poor hippocampal-PFC signaling could underlie contextual memory deficits and conditioned fear responses lacking context sensitivity in PTSD (Acheson et al. 2011). Considering the important control that the PFC exerts on fear and explicit memory function, as determined by the amygala and hippocampus respectively, it is important to consider the effect of noradrenergic modulation on PFC function. In this regard, non-selective β-receptor antagonism with propranolol has shown no effect on PFC function in monkeys, whereas the selective β_1-antagonist, betaxolol, was shown to enhance PFC function (Ramos et al. 2005). Since the PFC exerts inhibitory control over the hippocampus and amgydala (Shin et al. 2004), as well as the HPA axis, both of which exhibit exaggerated responses in PTSD (Radley et al. 2009), selective β_1-antagonism could offer greater control over traumatic memories and improved context sensitivity both in the context of PTSD as well as in an animal model of PTSD. Possibly the use of a β_1-selective antagonist may have provided an improved response over that of propranolol, as described in the study by Cohen et al. (2011). Furthermore, Schutsky et al (2011) showed that memory retrieval is

dependent on β_2- but not β_1-adrenergic receptors, and that the β_2-receptor too is a critical mediator of the acute response to stress. Consequently, both β_1- and β_2-receptors have distinct roles in CNS signaling and cognition (Schutsky et al. 2011). In another recent study, the β_2-antagonist ICI 118551 but not the β_1-antagonist betaxolol blocked forced swim stress-induced cocaine reinstatement (Vranjkovic et al. 2011). It is evident therefore, that both β_1- and β_2-adrenoceptors play a decisive role in PFC function and in the consolidation and regulation of fear memory, with the current evidence suggesting that the β_2-receptor is involved in memory retrieval and in anxiety and stress-inducing processes, such as those elicited by forced swim stress, while the β_1-receptor is more involved in tasks requiring cognitve processes such as working memory. That both β_1- and β_2-adrenoceptors are important in fear conditioning is therefore clear, while both betaxolol and ICI 118551 in this study were found to be as effective as propranolol to attenuate contextual conditioned fear, thus reinforcing the status of these compounds as viable options for secondary treatment to prevent the development of PTSD.

While the findings of this study have confirmed earlier studies implicating an important role for β_1- and β_2- adrenoceptors in conditioned fear, and presenting with equal efficacy versus propranolol, no difference was evident between the effect of β_1- and β_2-receptor antagonism with respect to inhibiting the consolidation of conditioned fear. This reaffirms that conditioned fear consolidation is dependent on both these receptors. However, whereas the clinical efficacy of propranolol in the prevention and treatment of PTSD posttrauma is debated (Cohen et al. 2011a, McGhee et al. 2009, Sharp et al. 2010, Stein et al. 2007), evidence for distinct roles of the β_1- and β_2-receptors could suggest that targeting specific β-adrenergic receptors in PTSD might be a more promising symptom-specific treatment approach to treating stress related disorders, and that the application of these selective receptor antagonists might produce divergent effects both in fear conditioning and pathological animal models of PTSD, as well as in PTSD. Since β_1-receptor antagonism has been shown to improve PFC function (Ramos et al. 2005), selectively targeting the β_1-receptor in the aftermath of a traumatic event might improve PFC inhibitory

control over the amygdala and hippocampus (Shin et al. 2006), thus decreasing exaggerated fear responses. This in turn would improve context sensitivity to reminders of the trauma and prevent inappropriate fearful reactions characteristic of PTSD. On the other hand, a recent study has shown that memory retrieval is enhanced by β_2, but not β_1 adrenergic antagonism (Schutsky et al. 2011), so that selectively targeting the β_2 receptor posttrauma or even in established PTSD, might decrease the memory intrusions akin to PTSD. Further studies looking at these drugs in an active avoidance protocol, looking specifically at issues relating to fear sensitisation and extinction, may reveal more as to the specific role for these two receptors in fear learning and memory. This is especially relevant if one considers that active avoidance, as a form of cued fear conditioning, relies more on the amygdala, while passive avoidance relies strongly on hippocampal function.

A dose response design with drugs selectively targeting the α_1- and α_2-receptors showed that only the α_1-antagonist, prazosin, at a dose of 0.1 mg/kg was effective in attenuating conditioned fear consolidation. To the best of my knowledge, this is the first study describing the effects of prazosin in a passive avoidance protocol of this design as has been described here. Indeed, for this reason the observed dose could not be corroborated with any similar studies in the literature. Furthermore, this response was equal to that engendered by propranolol. The α_1-receptor has been shown to be critical in various memory formation and consolidation processes (Ferry et al. 1999a, Ferry et al. 1999b), while in the context of the current study, blockade of α_1-receptors has been shown to protect PFC function and reduce amygdala activity in animals (Ferry et al. 1999c), which would result in a diminished fear response (Shin et al. 2004, Shin et al. 2006). Indeed, prazosin has successfully been used to reduce trauma-related nightmares in patients with PTSD (Peskind et al. 2003, Raskind et al. 2003, Thompson et al. 2008). This study has therefore provided additional supportive evidence for the role of α_1-mechanisms in contextual memory storage such as those involved in inhibitory avoidance paradigm, and supports the clinical use of prazosin in PTSD, not only to prevent nightmares, but as a possible secondary treatment to prevent the development of PTSD.

The study also produced new data with respect to effects on fear conditioning on the selective α_2-agonist, guanfacine, and the α_2-antagonist yohimbine. In contrast with studies demonstrating the efficacy of α_2-agonists in impairing memory retention (Davies et al. 2004, Ferry, McGaugh 2008, Gibbs et al. 2010), guanfacine did not significantly impair aversive memory retention in the current study. Presynaptic α_2-adrenoceptors exert a tonic inhibitory influence on noradrenaline release, particularly during stress, with memory loss following activation of α_2-receptors in the locus coeruleus strongly supportive of this (Gibbs et al. 2010). Buffalari et. al (2007) also demonstrated that noradrenergic input to the BLA exerts a potent inhibitory action over spontaneous and evoked activity mediated by α_2-receptor activation, while excitatory effects are mediated by β-receptors. Based on these papers, the α_2-adrenoceptor should theoretically be an important site where modulation of learned fear can be targeted and altered. In line with the argument above regarding the role of the α_2-receptor in amydala activation and fear responding, the α_2-antagonist, yohimbine, tended to bolster the memory for the aversive event, albeit not significantly so. Indeed, yohimbine is well known to evoke anxiety in animals (Guerrero-Figueroa et al. 1972) and humans (Charney et al. 1983), while the ability of yohimbine to also promote memory consolidation is evidenced in other studies (Gibbs et al. 2010). Despite guanfacine not engendering any noteworthy effects on fear memory consolidation, this lack of efficacy could explain similar results obtained in recent trials to ameliorate the symptoms of PTSD (Davis et al. 2008, Neylan et al. 2006). Differential roles for the various subtypes of α_2-subreceptors, namely α_{2A}-, α_{2B}- and α_{2C}-subreceptors, have been demonstrated in tasks requiring cognitive processes (Krystal, Neumeister 2009, Sallinen et al. 1999, Schramm et al. 2001, Small et al. 2000). Furthermore, the amygdala has a higher density of α_2-receptors compared to α_1-receptors and selective blocking or activation of these receptors is expected to modulate memory storage (Ferry, McGaugh 2008). This suggests that targeting specific α_{2A}-, α_{2B}- and α_{2C}-subreceptors could be an attractive future option to investigate with respect to contextual fear conditioning, especially in conjunction with a translational animal model of PTSD. Again studies looking at the role of the α_1- and α_2-receptors in an active avoidance protocol may be

useful to obtain additional information regarding other critical areas of fear learning, since active avoidance is a form of cued fear conditioning, which is more reliant on the amygdala than the hippocampus.

To conclude, this study has provided valuable new information on the role of various noradrenergic receptors in a passive avoidance model of conditioned fear. These data have been obtained using a single animal model as well as an animal with a naturally enhanced sensitivity to stress, thus increasing the overall validity and reliability of the findings. Moreover, these findings have contributed significantly to current knowledge regarding the neurobiology underlying conditioned fear memory and how these processes can be pharmacologically targeted to alter the course of a traumatic emotional experience. The study therefore has significant relevance to especially PTSD and for using drugs that can prevent the development of PTSD following an acute emotional experience.

The study has also conferred valuable predictive validity to the Gemini^{TM} model of passive avoidance which can now be applied in future exploratory studies in pathological animal models of stress and anxiety. The knowledge obtained from this study can now be applied to a translational animal model of PTSD to investigate whether selective receptor antagonists and agonists, which effectively attenuated fear conditioning in a non-pathological model as has been described here, would exert similar effects on conditioned fear responses in a PTSD-like paradigm, eg TDS or SPS.

The primary observations and conclusion drawn from this study may be summarised as follows:

- Propranolol 10 mg/kg significantly attenuates the consolidation of conditioned contextual fear when administered immediately after exposure to the footshock-context pairings, and is dependent on a central mechanism of action.

- Selective targeting of β_1-, β_2-, and α_1-receptors with betaxolol 10 mg/kg, ICI 118551 1 mg/kg and prazosin 0.1 mg/kg respectively, impairs the consolidation of conditioned contextual fear in a measure comparable to the effect elicited by propranolol 10 mg/kg.

- α_2-agonism with guanfacine was not effective in attenuating contextual conditioned fear, whereas α_2-antagonism with yohimbine tended to bolster conditioned fear consolidation.

- β_1- and β_2-receptors are equally important in the laying down of fear memory, as assessed in a passive avoidance model, thus suggesting that selective β_1- or β_2-receptor active drugs are viable candidates for application in the secondary prevention of PTSD.

- This study supports the use of propranolol in the secondary prevention of PTSD, although its efficacy may be dose-dependent.

- The α_1-adrenoreceptor occupies a central role in fear memory consolidation, while the study has also confirmed the status of prazosin as an effective drug in the treatment of PTSD (e.g. nightmares), but also as an alternative to propranolol in the secondary prevention of the disorder.

4.2 SHORTCOMINGS OF THIS STUDY

- Apart from the data presented in Addendum A confirming that the inhibitory effects of propranolol on fear memory is a centrally mediated response, this study did not provide similar definitive proof that betaxolol, ICI 118551 and prazosin also exerted their effects exclusively via CNS mechanisms. Although they are all known to penetrate the brain, unlike nadolol, further study is possibly needed to confirm this assumption.

- This study employed cut-off criteria on the first and second day of passive avoidance training in accordance with current literature. However, the cut-off procedures followed in this study produced data

containing a number of outliers that placed limitations on the statistical interpretation of the data. Employing shorter cut-off times in future studies might limit this problem.

- Due to ethical considerations, and compounded by the applied cut-off criteria, the amount of animals per treatment cohort were limited which could have adversely affected statistical analysis of the data. For example, the lack of significant response with guanfacine, as well as the lack of an enhancement of fear responding with yohimbine, may well have benefited from a larger n-value. Using a larger number of subjects per cohort might therefore result in a more even spread of data resulting in more statistically meaningful results.

- This study investigated the role of noradrenergic receptors in a contextual conditioned fear paradigm with relevance to PTSD. However, due to time constraints, the drugs that demonstrated efficacy in attenuating conditioned fear could not be studied in a translational (pathological) animal model of PTSD, e.g. TDS. The conclusions made must therefore be viewed cautiously when attempting to extrapolate these findings to PTSD. Nevertheless, the knowledge obtained through this route can in future be studied in a suitable translational PTSD animal model in order to establish whether these drugs performed similarly in a healthy compared to a pathological model of conditioned fear.

- This study used step-through latency on the second day as a measure of memory retention. Due to the training trial on day 1 being used exlusively as a first exposure to the context and the acquisition of an association between the context and the unconditioned stimulus, latency data obtained from the training trial on day 1 was not included in data analyses to determine the measure of fear memory retention. In this regard, latency data for the retention trial on day 2 was used exclusively for this purpose, as has been the approach by others (Ferry et al. 1999b, Ferry, McGaugh 1999). An alternative way of processing the data by assessing the retention latencies on day 2

in terms of the training latencies on day 1 could be used in future studies and warrants future investigation. Future studies conducted with a larger n value and with shorter cut off times should also produce data with less outliers, which would provide greater practical significance to the data. However, a shortage of available time precluded such an investigation, and is recommended for future studies.

4.3 RECOMMENDATIONS FOR FUTURE STUDIES

- The study should be extended so that drugs are used in a sub-chronic post-training treatment design, to assess the effect of sub-chronic vs acute administration on the impairment of aversive conditioned memories in a non-pathological model. Further, this response should be correlated with the effect in a pathological animal model.

- The neurobiology of a stress disorder, such as PTSD, is characterised by very specific dysfunctional and maladaptive neurobiological systems. The effect of drug intervention on conditioned fear in a pathological model could possibly produce results that are not congruent with findings described in a non-pathological model, as is the subject of this study. The following future studies are therefore warranted:

    - Reassess the possible benefits of post-training administration of the selective β_1-, β_2-, α_1-, and α_2-adrenergic drugs employed in this study but now in a pathological animal model of PTSD, in order to investigate possible posttrauma treatment approaches in the prevention of the development of the disorder in susceptible populations. Further studies should apply the effective doses of these drugs in an animal model of PTSD.

    - Within this pathological model, the effect of time on contextual conditioned fear retention should be determined in the same acute administration paradigm. The retention test, however, should be performed in different cohorts at different time periods

after fear conditioning, ranging between 24 hours to an extended period beyond e.g. 1-3 weeks. In fact, the criterion for PTSD is that the symptoms should develop in severity over time and also be present for at least a month.

- Sub-chronic administration of these drugs should be applied in a pathological model and the retention of conditioned fear should be tested at different time intervals as stated above, to assess the benefits of these drugs on long-term retention of fearful contextual memory retention.

- In view of the lack of efficacy of the α_2-agonist guanfacine to attenuate conditioned fear, the effect of targeting more selective α_2-subreceptors in a pathological and non-pathological model should be determined.

- Since PTSD is characterized by enhanced conditioned fear responses which lack context sensitivity, the recollection of the specific context can be determined in a non-pathological and pathological animal model of PTSD. This might involve submitting the animal to the conditioned fear training trial as described in this dissertation, but hereafter exposing different cohorts to slightly different contexts: one cohort can be returned to the same context as described in this study; another cohort might be exposed to a different context (different apparatus with distinctly different features) but also with a light and dark compartment. This study would then determine if the animal retains aversive memory for the specific context or for a dark context in general, and might provide further validation of the animal model of PTSD.

- Further studies looking at these drugs in an active avoidance protocol, looking specifically at issues relating to fear sensitisation and extinction, may reveal more as to the specific role for these two receptors in fear learning and memory. Indeed, active avoidance is a form of cued fear conditioning, which is more dependent on amygdala activation and less dependent on the hippocampus (see section 2.3.2.2 and 2.3.3), while passive avoidance is a form of contextual fear conditioning, relying more

on hippocampal function. Since both the amygdala and hippocampus show structural and functional abnormalities in PTSD, employing these drugs in an active avoidance paradigm might give greater insights into the role of the selective receptors involved in symptoms related to dysfunctional amygdalar signaling, such as avoidance, intrusive memories and exaggerated startle responses.

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Addendum A

The centrally active β-antagonist propranolol, but not the peripherally active β-antagonist nadolol, prevents fear learning in a passive avoidance protocol: a pilot study validating the Gemini™ Avoidance System

A.1 INTRODUCTION

Fear conditioning is a behavioural paradigm that uses associative learning to study the neural mechanisms underlying learning, memory and fear (LeDoux 1992, Maren 2001). Fear conditioning occurs when a neutral or conditioned stimulus (CS) is paired with an aversive or unconditioned stimulus (US), eliciting fearful responses to the neutral CS due to an anticipation of the US even in the absence of the US (Rescorla 1968, Watson, Rayner 1920). This paradigm is discussed in detail in section 2.3 of this dissertation.

Fear conditioning is used to study the neurobiology, neurocircuitry and etiology of various anxiety disorders and disorders involving cognitive processing. It is also a valuable technique with which to investigate possible treatment approaches that may have value in alleviating disorders characterized by exacerbated conditioned fear (De Oca et al. 1998, Kim et al. 1993, Kim, Jung 2006). In PTSD, a traumatic event (US) usually coincides with cues such as sounds, smells, sights, specific contexts etc. (CSs) that were present during the occurence of the traumatic event, and these CSs become associated with the US (traumatic event), later giving rise to some of the primary symptoms of the disorder, including flashbacks, nightmares, memory intrusions and a tendency to show exaggerated conditioned fear responses (American Psychiatric Association 2000). This phenomenon is analogous to fear conditioning, which

models this learned fear paradigm. Fear conditioning can therefore be applied in animal models of PTSD and other anxiety disorders to investigate numerous factors related to the illness, including pharmacological interventions and underlying neurobiology.

Passive avoidance, also referred to as inhibitory avoidance, is a form of contextual fear conditioning which uses the animal's natural preference for a dark area to test aversive learning and subsequent behavioural adaptation (Porciúncula et al. 2002). It involves taking an animal and placing it in an illuminated chamber which is connected to a dark chamber by a guillotine door. Upon entering the dark chamber, a footshock (US) is administered to the animal. If the US is severe enough, the subject will rapidly learn to associate this preferred dark context with the aversive footshock stimulus, and thus display an aversion to enter this compartment upon subsequent re-exposure 24 hours later (Blanchard, Blanchard 1969). The latency to cross over into the dark chamber (step-through latency or STL) is used as an indicator of learning and memory – a higher STL indicates robust learning and memory for the aversive association between the context and footshock (Ohno, Watanabe 1996).

The noradrenergic system is an important mediator of the fear response. Experiences eliciting strong emotional responses are transformed into very strong memories (McGaugh 2002), and these experiences are associated with increased noradrenaline levels in the brain areas involved in fear learning and memory, most notably the amygdala (McGaugh et al. 2002, McGaugh 2004, McIntyre et al. 2002). A large body of evidence supports the role of noradrenergic receptor activation in the amygdala as a crucial mediator for fear memory consolidation (Ferry et al. 1999a, Ferry et al. 1999b, Ferry, McGaugh 1999, Liang et al. 1986, Liang et al. 1990), while emotionally aroused noradrenergic activation of the basolateral amygdala (BLA) has been shown to strengthen memory consolidation in the hippocampus (McGaugh 2004), the latter being strongly associated with contextual memory related to fear conditioning (Anagnostaras et al. 1999, Kim, Fanselow 1992, Maren et al. 1997). Furthermore, administration of a footshock to rodents, such as is used in passive avoidance, induces the release of noradrenaline in the amygdala in a measure which can be directly correlated with the intensity of the shock (Galvez

et al. 1996, Quirarte et al. 1998) as well as the measure in which the aversive memory is retained (McIntyre et al. 2002).

Propranolol is a centrally active, non-selective β-adrenoceptor antagonist. It suppresses noradrenergic activity in the brain areas involved in fear conditioning by inhibiting the activation of the adenylate cyclase-cAMP pathway, thereby inhibiting the formation of PKA (McGaugh, Roozendaal 2002), an important factor implicated in LTP (Huang, Kandel 1998). This results in inhibition of cAMP response element binding protein (CREB) formation (Carew 1996), and thus impedes protein synthesis, an essential prerequisite for memory formation (Josselyn et al. 2001). This pathway is discussed in more detail in section 2.3.4.5 of this dissertation. Memory processing takes place in distinct stages, namely acquisition, consolidation and reconsolidation and retrieval (Milner et al. 1998). The *acquisition* stage occurs when the animal learns to associate a cue or context with an impending aversive event, such as a footshock, and this is stored as a labile memory. *Consolidation* describes the process during which this labile memory assumes a more fixed state, a process requiring protein synthesis and which can last for hours or even days (Davis, Squire 1984). During memory consolidation short-term memory is transformed to long-term memory, which renders the memory increasingly more resistant to further manipulation. *Reconsolidation* forms part of the consolidation process, but occurs when the CS and US is presented again to re-inforce the associative memory. In PTSD flash backs and memory intrusions result in reliving of the trauma in such a vivid manner that these symptoms very often cause the repeated reconsolidation of fearful memories, resulting in a strongly reinforced memory trace. *Retrieval* is the stage where the actual retention or recollection of the memory of the CS-US association takes place (Abel, Lattal 2001). Propranolol has been shown to effectively impair the acquisition (Do Monte et al. 2008, Stern et al. 2008), consolidation (Cahill et al. 2000, Ferry, McGaugh 1999, Grillon et al. 2004, Kroon, Carobrez 2009, Roozendaal et al. 1999), retrieval (Ouyang, Thomas 2005, Rodriguez-Romaguera et al. 2009) and reconsolidation (Dębiec, Ledoux 2004, Przybyslawski et al. 1999) of fearful memories in various paradigms of learning. The extent to which a memory is consolidated can be measured with a retention test, during which a reminder of

the event is produced in the form of a cue or a similar context. Retention for the memory can be measured by the animal's behaviour in the form of freezing or avoidance of the cue or context associated with the aversive event. The measure of consolidation can be directly correlated with the retention of the memory for the event, and is often expressed as retention. In the passive avoidance paradigm, the step-through latency (STL) is used as a measure of retention of the aversive memory, and is used to study the effect of certain interventions on memory consolidation.

The criteria for validating an animal model have been discussed in section 2.3.6. In this pilot study, the modulation of fear memory consolidation with two selected β-adrenergic antagonists was assessed, viz, propranolol and nadolol. Due to its established role in impairing conditioned fear memories, propranolol was employed posttraining as a form of predictive validation of the passive avoidance model under our laboratory conditions. Moreover, this validation was extended to demonstrate that central and not peripheral β adrenergic receptor antagonism is required for suppression of fear memory. In order to confirm this assumption, a non-centrally acting β-blocker, nadolol, was compared head-to-head with propranolol. Indeed, nadolol does not cross the blood-brain barrier and exerts no central β-adrenergic effect, although both nadolol and propranolol have similar potencies as non-selective β-receptor antagonists (Escoubet et al. 1986). A different role for centrally vs peripherally acting β-blockers in memory-related tasks has previously been established (van Stegeren et al. 1998). Both drugs underwent a three-tiered dose response analysis using the Gemini^{TM} Avoidance System in order to establish an effective dose under our conditions of study. In all instances, n=10 was the minimum number of rats per group. I proposed that the centrally active β-adrenergic antagonist, propranolol, but not the non-centrally active β-adrenergic antagonist, nadolol, would effectively attenuate fear conditioning in this passive avoidance paradigm. Furthermore, propranolol would demonstrate a distinct dose-dependent response with the most effective dose being selected as reference standard in the main drug treatment study (see Chapter 3).

A.2 MATERIALS AND METHODS

A.2.1 ANIMALS

Male Wistar rats, weighing 180-230g, were selected for this study since these animals have been described as showing more robust conditioned and unconditioned responses to stress (Staples, McGregor 2006), thus improving the overall sensitivity and validity of the behavioural tests. Animals were housed in groups of five in standard laboratory cages (380 mm x 380 mm x 320 mm) at the Animal Research Centre of the North-West University under well-maintained environmental conditions, including temperature (21 ℃), and relative humidity (50±5%). A positive air pressure with air filtration 99.7% effective for a particle size of 2 micron and 99.9% for a particle size of 5 micron, as well as a 12 hour light/dark cycle was maintained, with lights off at 18h00. Animals were handled according to the guidelines stipulated by the Ethics Committee of the North West University (Ethics Approval Number: NWU-00007-11-S5).

A.2.2 DRUGS

Propranolol hydrochloride and nadolol base were purchased from Sigma Aldrich in Johannesburg and dissolved in sterile water for injection to obtain the desired concentration. Both drugs were injected at a volume of 1 ml/kg. Since propranolol has a higher first pass metabolism compared to nadolol (Borchard 1990, McDevitt 1987), and in order to reduce possible differences in peak blood concentrations, both drugs were administered subcutaneously (sc). Drugs were injected immediately after the passive avoidance training on day one of testing. Propranolol and nadolol were tested over a 3-tiered dose response analysis to establish the most effective doses under our laboratory conditions. According to earlier studies, propranolol was tested across a dosage range of 5; 10 and 20 mg/kg (Cahill et al. 2000, Do-Monte et al. 2010, Ebrahimi et al. 2010), while nadolol was administered across a dosage range of 2; 10 and 20 mg/kg (Ebrahimi et al. 2010, Robinson, Franklin 2007).

A.2.3 APPARATUS AND BEHAVIOURAL ASSESSMENT

A.2.3.1 Passive avoidance fear conditioning

Passive avoidance sessions were conducted using the computer-assisted GEMINI™ Avoidance System (San Diego Instruments, San Diego, CA, USA). The GEMINI consists of two equal chambers separated by a computer-automated guillotine gate. The walls of the chamber are made of black Plexiglas and the stainless steel grid floor consists of rods that are capable of administering footshocks to the animal. Each chamber has 8 photobeams that can detect the exact location of the animal so that a computer-controlled protocol can be employed. A one trial step-through procedure was used to measure the effect of the various drugs and doses on the consolidation of fearful learned memories acquired after a single footshock was paired upon entrance into the dark chamber. The procedure that was followed was based on previously described studies employing the same passive avoidance paradigm (Díaz-Trujillo et al. 2009, Khakpour-Taleghani et al. 2008, Nielson et al. 1999, Piri, Zarrindast 2011). During the training trial on day one of behavioural testing, the animal was placed in one of the compartments with the gate closed and allowed free exploration. After 30 seconds, this compartment lit up and the guillotine gate opened. When the animal crossed over to the dark compartment, the latency (or time in seconds) was recorded and the gate was closed, whereafter a mild footshock of 0.8 mA was applied for 5 seconds. Similar shock intensities and duration of shock have been well-described in the literature (Acosta et al. 2000, Bainbridge et al. 2008, Díaz-Trujillo et al. 2009, Jafari-Sabet 2006). The animal was then left in the dark chamber for an additional 10 seconds to allow for the association between the footshock and the context (dark compartment) to be formed. After removal of the animal from the apparatus, the testing chambers were cleaned with 98% absolute ethanol to remove any odour trails. In accordance with the selection of animals discussed in section 2.3.7, to capture the more stress sensitive animals for the study, cut-off behavioural criteria were applied by removing animals that exceeded a latency of 60 s to enter the dark compartment in the training trial and replacing it with a naive animal, ultimately aiming for the inclusion of 10 animals per

treatment group (Browman et al. 2005). Following these procedures, each individual animal was then removed from the apparatus whereupon each received the specified drug treatment by subcutaneous injection, in a final volume of 1 ml/kg. Control animals received subcutaneous injections of saline at a volume of 1 ml/kg. Twenty-four hours later, retention of the aversive memory was tested. The procedure was repeated with the exception that no shock was administered upon entrance into the dark compartment. A cut-off time of 600 s was applied in the retention trial according to similarly conducted studies (Díaz-Trujillo et al. 2009, Khakpour-Taleghani et al. 2008, Liu et al. 2009, Nielson et al. 1999). All experiments were conducted between 7h00 and 13h00 during the light cycle.

A.2.3.2 Locomotor assessment

Since this fear conditioning procedure is dependent on the movement of the animal, the outcome might be affected by anomalies in the inherent locomotor activity of the animals. Therefore, to account for this, horizontal activity was assessed for 5 minutes on both days, five minutes prior to fear conditioning, and before drug dosing on day one. Horizontal activity was measured as the distance walked in cm. General locomotor activity was assessed using the Digiscan Animal Activity Monitor (*Omnitech* Electronics, Columbus, OH, USA). Each monitor consisted of a 42 x 42 x 30.5 cm clear Plexiglas box with a grid of 16 infrared beams 2.5 cm apart from front to back and from side to side. Monitors were connected to a Digiscan Analyzer that collated the transmitted activity data in digital form with subsequent analysis by a personal computer.

A.2.4 EXPERIMENTAL DESIGN

Animals were randomly divided into drug treatment cohorts with 10 animals per treatment group. Saline was used as control. Behavioural assessments were done over two days: on the first day, locomotor activity was assessed for five minutes, followed by a single passive avoidance training trial. After passive avoidance training, drugs were administered by subcutaneous administration

and animals returned to their home cages. Twenty-four hours later, locomotor activity was re-assessed as on the first day, and the passive avoidance retention trial was conducted with a cut-off time of 600 seconds. No drugs were administered after the retention trial.

A.2.5 STATISTICAL ANALYSIS

In accordance with earlier work (Ferry et al. 1999b, Ferry, McGaugh 1999), only the step-through latencies as measured on day 2 are used for statistical analysis. Mean training latencies for the different treatment cohorts are reported but were not used for statistical analyses. Comparisons among mean retention latencies on day 2 for the different treatment groups were performed with one-way analyses of variances (ANOVA) to determine if statistically significant differences existed between the means of the step-through latencies. Levene's tests were performed to assure equality of variances in each ANOVA. In the case of unequality of variances, Welch tests were performed since the assumption for normal ANOVA was not met. Normal probability plots on the residuals were done to assure that the data was fairly normally distributed (Tabachnick, Fidell 2001). Post hoc Dunnett's tests were done to determine whether significant differences were evident between the means of test groups and saline controls on day 2.

To determine if inherent locomotor activity for all the groups were comparable to saline control, an ANOVA was performed on locomotor activity on day one immediately before drug treatment and passive avoidance training. Locomotor activity on day two immediately prior to behavioural testing was analysed in like fashion. However, in the event that statistically significant group differences were found on day one (with an ANOVA), an ANCOVA was performed on locomotor activity data on the second day with locomotor activity on day one used as the covariate. Adjusted means yielded by the ANCOVA were then used to perform Dunnett's tests to determine differences in locomotor activity on day two between all groups compared to saline treated groups. The same precautions regarding assumptions for these procedures, as described in the paragraph above, were performed.

These procedures were done using the statistical data analysis software systems STATISTICA (StatSoft, Inc. 2007) version 8.0 and SAS (SAS Institute Inc. 2005). All tests were done at a 0.05 significance level.

A.3 RESULTS

A.3.1 PASSIVE AVOIDANCE

Training latencies on day 1 for all treatment cohorts before administration of saline or non-selective β-antagonists were below 60 s due to the 60 s cut-off time and the mean ± SEM training latencies are shown in table 1. 9.5% of subjects did not meet cut-off latency criteria on day 1 and were removed from the study. Exact retention latency and p-values for the retention trial on day 2 are shown in Figure 1 and summarised in Table 2.

Table 1: Mean training latencies in a step-through passive avoidance task for all treatment cohorts prior to treatment with either saline, the central β-adrenergic antagonist, propranolol (5; 10 and 20 mg/kg) and the peripheral β-adrenergic antagonist, nadolol (2; 10 and 20 mg/kg), on passive avoidance acquisition latencies. These data was not used for statistical analyses, as stated in the text. n=10 for each group.

TREATMENT COHORT	Mean Training Latency (s) (Mean±SEM)
Saline	29.91 ± 4.24
Propranolol 5 mg/kg	9.80 ± 1.81
Propranolol 10 mg/kg	22.35 ± 3.55
Propranolol 20 mg/kg	14.67 ± 2.38
Nadolol 2 mg/kg	11.93 ± 2.23
Nadolol 10 mg/kg	14.36 ± 2.21
Nadolol 20 mg/kg	16.68 ± 2.59

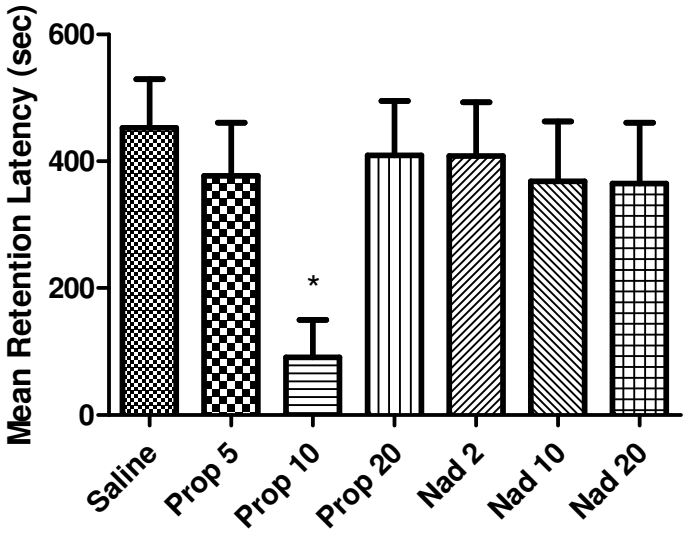

Fig 1 Retention latencies (mean ± SEM; sec) in the step-through passive avoidance task in rats treated with either saline (control), propranolol (Prop) 5; 10 or 20 mg/kg, or nadolol (Nad) 2; 10 or 20 mg/kg. n=10 for each group. *p<0.05 compared to saline control group (ANOVA and Dunnett's post hoc).

Table 2: Effect of post-training administration of the central β-adrenergic antagonist, propranolol and the peripheral β-adrenergic antagonist, nadolol, on passive avoidance retention latencies. Values are expressed as mean ± SEM of 10 animals per group. Dunnett's p-values for each drug treatment compared to saline is given.

DRUG TREATMENT	Mean Retention Latency (s) (Mean±SEM)	Dunnett's post hoc p-value (compared to saline control)
Saline	453.18 ± 76.77	
Propranolol 5 mg/kg	377.08 ± 83.62	0.971
Propranolol 10 mg/kg	90.93 ± 58.61	**0.017**
Propranolol 20 mg/kg	409.28 ± 85.88	0.998
Nadolol 2 mg/kg	408.15 ± 84.86	0.998
Nadolol 10 mg/kg	368.24 ± 94.65	0.951
Nadolol 20 mg/kg	364.79 ± 96.03	0.942

The mean retention latency of the saline control group was 453.18 ± 76.77 s, compared to a mean training latency of 29.91 ± 4.24 s,I ndicating that the footshock induced strong retention of the inhibitory avoidance training.

Dunnett's post hoc test revealed that post-training administration of propranolol 10 mg/kg produced a significant deficit in retention of the aversive memory (Table 2, 90.93 ± 58.66 s; p=0.017). In contrast, post-training administration of propranolol 5 mg/kg and 20 mg/kg showed no significant effect on retention latencies (Table 2, 377.08 ± 83.62 s and 409.28 ± 85.88s respectively; n.s.). Post-training administration of the non-centrally acting β-antagonist, nadolol, produced no impairment on memory retention and the mean retention latencies did not show significant differences compared to saline (Table 2, nadolol 2 mg/kg 408.15 ± 84.86 s, p=0.998, n.s.; nadolol 10 mg/kg 368.24 ± 94.65 s, p=0.951, n.s.; nadolol 20 mg/kg 364.79 ± 96.03 s, p = 0.942, n.s.).

A.3.2. LOCOMOTOR ACTIVITY

ANOVA of locomotor activity between all groups on the first day versus saline (before drug treatment) showed significant differences between the different treatment groups, with a p-value of 0.019. Subsequently, locomotor activity on day two was analysed by ANCOVA using locomotor activity on day one as the covariate. The resulting adjusted means for locomotor activity on the second day are summarised in Table 3. Dunnett's post hoc test showed no statistically significant differences between the adjusted means of drug treatment versus saline treatment groups on day two. The saline treatment group showed an adjusted mean locomotor value of 641.59 cm, while locomotor activity for all treatment groups showed no statistically significant differences from saline. However, the propranolol 20 mg/kg group showed a locomotor activity of 298.26 cm, which was much lower than the distance walked by the other treatment groups and barely missed statistical significant difference compared to saline control, with p = 0.05. The p-values for the Dunnett's post hoc test are shown in Table 3, while figure 2 graphically depicts the effect of drug treatment on locomotor activity on the second day. However, the latter data are expressed as the adjusted means hence the absence of error bars.

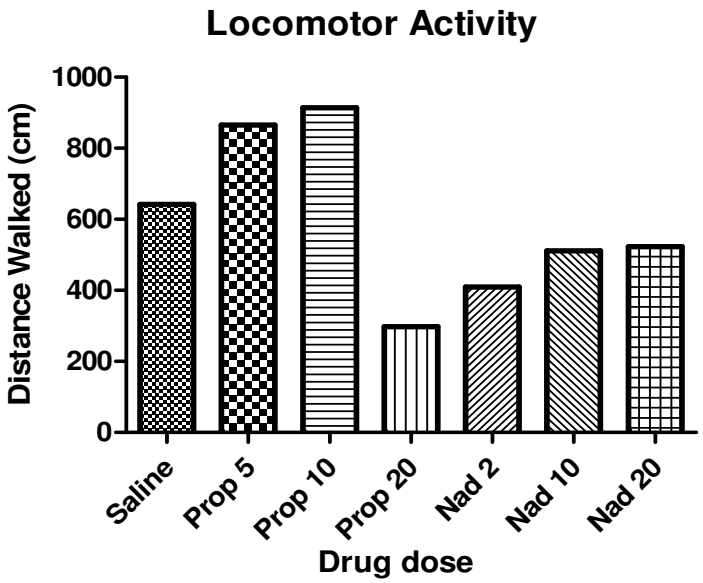

Fig 2 Locomotor activity for saline, propranolol (Prop) 5, 10 and 20 mg/kg and nadolol (Nad) 2, 10 and 20 mg/kg compared to saline, on day two 24 hours after drug treatment, expressed as the adjusted mean (corrected for differences in locomotor activity on day one) using ANCOVA and Dunnett's post hoc test.

Table 3 Locomotor activity on the first day before drug treatment and behavioural testing, and on the second day before behavioural testing, 24 hours after drug treatment expressed as adjusted mean (cm) ± SEM. Individual drug versus saline data were analysed using an ANCOVA and Dunnett's post hoc test.

DRUG TREATMENT	Distance walked (cm) (Adjusted means)	Dunnett's post hoc (p-values)
Saline	641.59	
Propranolol 5 mg/kg	864.73	0.3442
Propranolol 10 mg/kg	913.64	0.1284
Propranolol 20 mg/kg	298.26	0.0508
Nadolol 2 mg/kg	409.77	0.2586
Nadolol 10 mg/kg	511.10	0.7943
Nadolol 20 mg/kg	522.72	0.8373

A.4 DISCUSSION

The most noteworthy observations made in this study were that the non-selective β-receptor antagonist, propranolol, significantly and dose-dependently inhibited memory consolidation for a fear-related passive avoidance task at a dose of 10 mg/kg, without adversely affecting locomotor performance. Importantly, the non-CNS penetrant, non-selective β-receptor antagonist, nadolol, was ineffective in attenuating fear memory consolidation at all doses tested. Nadolol did not affect general locomotor activity at any dose.

Propranolol was found to be effective at a dose of 10 mg/kg compared to saline treated animals (Fig. 1), which corresponds well with that described in the current literature on effective doses of propranolol to impair the consolidation of fearful memories (Nielson et al. 1999, Przybyslawski et al. 1999). Importantly, no effect on locomotor performance was observed at this dose (Fig. 2). Interestingly, using a shock intensity of 0.5 mA for 5 s, Schneider et al. (2011) showed that propranolol 10 mg/kg was not effective in impairing conditioned fear at this dose unless an additional stressor (swim stress) was added (Schneider et al. 2011). The authors argue that propranolol's efficacy to impair conditioned fear memory is dependent on the intensity of the US, i.e. the footshock, and that their data suggests that this is not enough, requiring an additional swim stress with the footshock to be effective as a US. Based on the data presented in this pilot study, the lack of effect that the above authors described may very well be because the shock intensity of 0.5 mA for 5 s employed by these authors is not an adequate enough aversive stimulus necessary to illicit US-CS pairing. Moreover, the 0.8 mA, 5 s shock applied in the current study represents a more meaningful stressor that does not require an additional stressor. This is an interesting finding because it confirms propranolol's role in attenuating the consolidation of emotional memories, leaving memories which are not so emotionally loaded, intact (Cahill et al. 1994, Nielson et al. 1996, van Stegeren et al. 1998).

According to our data, the 5 mg/kg dose of propranolol did not show any significant impairment of fear memory consolidation compared to saline treatment (see Table 1) and without residual motor impairment (Fig 2), which contrasts with previous studies done at a similar dose (Nielson et al. 1999, Cahill et al. 2000). However, Cahill et al. (2000) found that a 5 mg/kg dose only impaired memory retention in a group of "good learners", and did not show the same robust effect in a group of "bad learners". Furthermore, Nielson et al. (1999) found that chronic administration of propranolol dose-dependently impaired memory retention, with doses of 2, 4, 8 and 12 mg/kg found to be effective. Considering these data, and in the light of data presented in this pilot study, it seems reasonable to conclude that a 5mg/kg dose of propranolol is insufficient to cause adequate β-adrenergic blockade necessary to impair fearful memory formation in rats, at least under the current conditions of study, and is thus not suitable for further consideration in the main study.

The 20 mg/kg dose of propranolol was not effective in impairing conditioned fear (Table 1). This was an unexpected finding since earlier studies have noted that propranolol shows a dose-dependent impairment of memory consolidation (Nielson et al. 1999). However, since this task is highly dependent on the integrity of movement in the animal, it is possible that these results may be adversely influenced by the lower inherent locomotor activity observed in this treatment group (Fig. 2). However, this trend towards lower locomotor activity was not shown to differ significantly from that of the saline treatment group (Table 2). Propranolol has a half-life of less than 1.5 hours in the rat and would be eliminated before the retention test on day 2 (24 h later) (Kim et al. 2001). Theoretically it is thus unlikely that any possible sedative effects of this drug could have contributed to the increased retention latencies observed in this treatment group. Nevertheless, the combined latency and locomotor data suggest that 20 mg/kg propranolol is either ineffective in attenuating fear memory, or that said efficacy is compromised by adverse effects of this dose on locomotor activity. Consequently, this dose is not amenable for further consideration in the main study.

Nadolol showed no effect on memory consolidation at any dose tested (Fig. 1), while no effects on locomotor activity were evident (Fig. 2). Since it shares similar β-adrenergic blocking properties with propranolol (Escoubet et al. 1986), this confirms that the effect exerted by propranolol 10 mg/kg was due to a central mechanism, and that central β-adrenergic activation is required for the formation and consolidation of emotional memories. This finding is also reflected in a study in humans using nadolol or propranolol to attenuate emotional memory retention (van Stegeren et al. 1998). In this study, propranolol selectively blocked memory for an emotionally arousing story in humans, whereas nadolol showed no effect on emotional memory retention. Furthermore, studies that combined functional neuroimaging during emotional memory tasks with β-adrenergic antagonism showed that the activation of the amygdala during emotional arousal is diminished by β-adrenergic blockade (Strange, Dolan 2004). The current finding thus provides further support for the inability of nadolol to attenuate memory for an aversive event, due to its selective peripheral action on β-receptors. This pilot study has therefore confirmed the proposed hypothesis that the centrally active β-adrenergic antagonist, propranolol, but not the non-centrally active β-adrenergic antagonist, nadolol, impairs aversive memory retention in a passive avoidance paradigm, and that this effect can be attributed to a central mechanism of action.

Prolonged and excessive noradrenergic activation in the aftermath of a trauma has been linked to increased risk for the development of PTSD (Vaiva et al. 2003), possibly through increased conditioned fear learning (Orr et al. 2000). A number of studies have reported the efficacy of propranolol in preventing the onset of PTSD (Pitman et al. 2002, Vaiva et al. 2003). Studies in animals have demonstrated similar findings (Ouyang, Thomas 2005, Rodriguez-Romaguera et al. 2009) in preventing the onset of PTSD-like symptoms, although a recent paper by Cohen et al (2010) did not support this. The latter study investigated the effect of immediate administration of propranolol in rats after exposure to predator scent stress, an animal model of PTSD. Propranolol failed to attenuate the persistent anxiety-like behaviours observed after exposure to intense

stress, and the authors propose that conditioning paradigms might not entirely model trauma-related responses. Their findings seem to echo sentiment regarding propranolol's efficacy in PTSD as well, with clinicians arguing that propranolol only has modest benefits as a means of secondary prevention of the disorder (Zohar et al 2011). Nevertheless, clinical evidence concurs that the drug has benefit, so that the relevance of effectively using propranolol to validate the Gemini^{TM} passive avoidance protocol is an important component of the study. Contemporary theories of PTSD concur that learning and memory processes such as fear conditioning underlie many of the avoidance and related symptoms of PTSD (Michael et al. 2005, Pitman 1989, Rothbaum, Davis 2003). It is therefore valuable to perform fear conditioning studies using novel treatment strategies in order to investigate new and more effective treatment strategies in PTSD.

To conclude, substantial evidence supports the efficacy of β-blockers to inhibit memory consolidation and emotional memory storage in animals (Barros et al. 2001, Gallagher et al. 1977, Ikegaya et al. 1997, Roozendaal et al. 1997)(Cahill et al. 1994, Roozendaal et al. 2004) and humans (Grillon et al. 2004, Harmer et al. 2001, Pitman et al. 2002). Data described here are therefore in line with these findings. This constitutes a veritable validation of the Gemini^{TM} Avoidance System for use in our laboratory and especially in later studies undertaken in this project (see Chapter 3) that will explore the effect of select pharmacological interventions targeting the noradrenergic system. Moreover, this work has not only confirmed that targeting central and not peripheral noradrenergic receptors is key to the efficacy of β-blockers in attenuating fear memory, but has also clearly delineated the dose of propranolol (10 mg/kg) that will be used as reference agent in the main treatment study described in Chapter 3. Finally, locomotor activity was routinely assessed in this study and has provided valuable additional information. Although a number of studies include this test in pharmacological studies in passive avoidance testing (Khakpour-Taleghani et al. 2008, Liu et al. 2009, Monleón et al. 2002, Soares et al. 2006), this is not always the case (Acosta et al. 2000, Eidi et al. 2003, Ferry, McGaugh 2008, Ohno, Watanabe 1996, Zarrindast et al. 2002). It can be argued that, from a

pharmacokinetic point of view, a single acute dose of these drugs will not markedly affect locomotor performance 24 hours later and as such influence latency testing in the passive avoidance paradigm (Kim et al. 2001). Since the dose selected for the main study based on this pilot study, i.e. 10 mg/kg, was found not to affect general locomotor performance, it was decided not to routinely undertake locomotor testing in the main study (Chapter 3).

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Addendum B

Authors' Instructions: Psychopharmacology

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Negotiation research spans many disciplines (Thompson 1990).
This result was later contradicted (Becker and Seligman 1996).
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Med. doi:10.1007/s001090000086

Book

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Book chapter
Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Doe J (1999) Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. Available via DIALOG. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999

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Please include in each file the following information: article title, journal name, author names; affiliation and e-mail address of the corresponding author. To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.

Audio, Video, and Animations

Always use MPEG-1 (.mpg) format.

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Spreadsheets

Spreadsheets should be converted to PDF if no interaction with the data is intended. If the readers should be encouraged to make their own calculations, spreadsheets should be submitted as .xls files (MS Excel).

Specialized Formats

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Collecting Multiple Files

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Numbering

If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables. Refer to the supplementary files as "Online Resource", e.g., "... as shown in the animation (Online Resource 3)", "... additional data are given in Online Resource 4". Name the files consecutively, e.g. "ESM_3.mpg", "ESM_4.pdf".

Captions

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