

Pharmacological evaluation of an alpha2C selective antagonist in an animal model of posttraumatic stress disorder

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I dedicate this dissertation to my family. A special feeling of gratitude to my loving family whose words of encouragement and love is what carried me these 2 years. None of you ever left my side and are very special to me.

ABSTRACT

Posttraumatic stress disorder (PTSD) is a psychiatric disorder that can manifest following the experience or witnessing of a life-threatening event such as military combat, natural disasters, terrorist incidents, serious accidents, or physical or sexual assault during child- or adulthood. Although most survivors of a traumatic event recover over time, approximately 30% of victims will go on to develop PTSD. Dysfunctional activity of multiple neurobiological pathways has been implicated in the pathology underlying PTSD symptomatology, including the noradrenergic, serotonergic, dopaminergic and glutamatergic systems, as well as the Hypothalamic-pituitary-adrenal (HPA) axis. These systems are also mutually interlinked, with dysfunction of one system affecting the function of the other, thereby complicating the pathology of the disorder. This complexity demands deeper investigation to define the roles of each system in the neuropathology of PTSD. The noradrenergic system is prominent and represents an important pharmacological target in attempts at preventing the development of PTSD in the immediate aftermath of trauma. In PTSD, it has been found that emotional events are associated with high levels of noradrenalin (NA) release in brain areas involved in learning and memory such as the amygdala and hippocampus. Emotional memories are mainly influenced by noradrenergic $\alpha_{1/2}$ and β_2 receptors. In the basolateral amygdala, emotionally aroused noradrenergic activation tends to strengthen memory consolidation in the hippocampus which is responsible for arranging contextual fear memory. Therefore, suitable curbing of the noradrenergic system could be an important neurobiological target in treating PTSD.

* * *

The first objective of this study was to validate the predator scent exposure (PSE) model in our laboratory and to determine if male Wistar rats will demonstrate different levels of anxiety akin to maladaptation and well-adaptation following exposure to a traumatic event, i.e. predator scent. In this regard, we identified the extreme ends of the normal distribution of anxious behaviour in response to PSE to validate the PSE model, as described below. That said, the number of faecal boli passed during PSE was used as a measure of immediate trauma-induced anxiety. To this end, the elevated plus maze (EPM) have been used to establish the application of the PSE model as a valid framework for novel drug discovery. Parameters assessed in the EPM included number of entries into the open (OAE) and closed (CAE) arms, as well as the time spent in the open (sOA) and closed (sCA). Head-dipping behaviour in the

open arms was applied as a measure of risk assessment and exploratory behaviour. Head dipping occurs in the central area of the maze and reflects an aversion to risk taking, which is related to the anxious state of the animal.

The PSE model has ethological relevance as it mimics intense stressful experiences and results in long term changes in behavioural, autonomic and hormonal responses that correlate with the symptoms in human PTSD. Regarding the conceptual validity of the model, “predator exposure trauma” is a potentially life-threatening situation and may represent a more “natural” challenge than other forms of stressors, i.e. electric tail shocks or restraint that may in fact be more related to extreme conditions such as torture. To confer validity to the model, male Wistar rats were used because of their recognised enhanced sensitivity to stress. Wistars were exposed individually under dim white light conditions (15 lux) for 10 minutes to a 10 cm x 10 cm cloth previously exposed to a male cat for 2 months, and a control group that were exposed to a clean non-cat-scented 10 cm x 10 cm cloth, with both groups being tracked and recorded digitally for subsequent analysis using Ethovision XT® software. Evidence from this study confirmed that at least 20 – 25% of the exposed group develop anxiety-like behaviours in the EPM when exposed to cat scent, while the remainder of the group was regarded to be resilient. This finding is important since individual variation in and susceptibility to trauma-related pathology is a key criterion for an animal model of PTSD. This effectively validated the animal model on face value in our laboratory for further application in this study.

* * *

Given the causal role for the hypothalamic-pituitary-adrenal (HPA) axis in PTSD, the second objective of this study was to compare plasmacorticosterone concentrations between treatment-naive well-adapted and maladapted animals as assessed 21 days post PSE. Moreover, we also sought to determine whether the selective α_{2C} AR antagonist, ORM-10921, was able to modify plasma corticosterone in PSE animals when dosed immediately post-PSE for 21 days. ORM-10921 treatment induced a subtle albeit insignificant trend towards hypocortisolemia however upon close scrutiny ORM-10921 treated animals were characterised by an increase in plasma corticosterone and an associated lowering of anxiety compared to vehicle treated cohorts.

* * *

Finally, considering the prominent role for NA in the neurobiology and treatment of PTSD, the third objective of this study was to investigate whether the selective α_{2C} AR antagonist, ORM-10921, is capable of reversing PSE-induced anxiety-like behaviour related to PTSD. Considering the less than adequate response of clinical PTSD to modulation of the noradrenergic and serotonergic systems, i.e. chronic treatment with *inter alia* TCAs, SNRIs, and SSRIs, as well as disappointing findings regarding the prophylactic use of propranolol in PTSD, there is increasing need for novel drug treatments that will offer improved and sustained efficacy. The current investigation found that administration of 0.3 mg/kg ORM-10921 only from 8 days *after* exposure (Addendum C) effectively increased behavioural disruptions evident in the PSE model, as evinced by a decrease in number open arm entries (OAE) and a decrease the time spent in open arms (sOA); this was also found in saline treated groups. Interestingly, ORM-10921 dosed in this manner tended to elevate corticosterone levels, which coincides with some evidence in the EPM that delayed-onset ORM-10921 administration is anxiogenic.

That said, administration of a 0.3 mg/kg immediately after exposure (Chapter 3) reduced behavioural disruptions that model PTSD, with chronic ORM-10921 treatment significantly lowering anxiety like-behaviours evinced by increases in the number of open arm entries(OAE) and time spent in open arms (sOA) compared to the saline treated animals. ORM-10921 also increased risk assessment and exploratory behaviour compared to saline treated animals as evident in the number of head dipping episodes.

Concluding, this study has conferred construct, face and predictive validity to the PSE model established in our laboratory and confirms the model's status as a prominent animal model in PTSD. Moreover, it has provided greater insight into the role of noradrenergic receptors in anxiety related PTSD by providing supportive evidence that selectively blocking the α_{2C} AR inhibits PTSD-related behaviour, notably so when administered immediately post-trauma. ORM-10921 therefore may be a viable secondary treatment option to prevent the development of PTSD post trauma. Further studies employing these novel agents in the treatment of anxiety disorders, such as PTSD, are encouraged, and will further our understanding of the role of α_{2C} AR in such disorders, as its viability as a therapeutic target.

Keywords: PTSD, Predator scent exposure model, α_{2C} AR antagonist, corticosterone concentrations, cat odour, elevated plus maze (EPM)

OPSOMMING

Post-traumatische spanningstoestand (PTST) is 'n psigiatriese versteuring wat presenteer nadat 'n slagoffer blootgestel is aan, of ten aanskoue was van 'n lewensbedreigende geval, bv. militêre skermutseling, natuurlike rampe, terroris-verwante insidente, motorongelukke of seksuele aanranding. Alhoewel die meeste van hierdie slagoffers oor tyd herstel, ontwikkel volslae PTST in ongeveer 30% van gevalle. Wanfunksionele aktiwiteit van 'n meerderheid neurobiologiese en neuroendokriene breinbane is al in die patologie onderliggend aan PTST gedemonstreer, insluitend abnormale noradrenergiese, serotonergiese, dopamienergiese en glutamatergiese sientransduksie, sowel as versteurde hipotalamus-pituitêre aksisfunksionering. Ook is hierdie sisteme onderling verwant aanmekaar, met afwykings in die een komponent wat versteurings in 'n ander kan veroorsaak. Hierdie ingewikkelde verwantskap noodsaak 'n grondige begrip van genoemde interaksies en die rol wat dit mag speel in die bemiddeling van PTST. Die noradrenergiese sisteem speel veral 'n prominente rol en kan dus moontlik as 'n belangrike farmakologiese teiken dien betreffende pogings om PTST onmiddelik na blootstelling aan akute trauma te voorkom. Daar is gevind dat in soverre PTST bestudeer word, emosionele gebeure geassosieer word met hoë vlakke van noradrenalin (NA) in die breinareas wat betrokke is by leervermoë en geheue; hierdie areas sluit bv. die amigdala en die hippocampus in. Die kliniese gravitas van emosionele geheue word hoofsaaklik deur noradrenergiese $\alpha_{1/2}$ - and β_2 -reseptorsbeïnvloed. So versterk emosioneel-gesnellerde NA-afskeiding in die basolaterale amigdala geheuevaslegging in die hippocampus wat op sy beurt verantwoordelik is vir die verwerking van kontekstuele vrees-verwante geheue. Dit sou daarom moontlik wees om trauma-blootgestelde individue te ondervang alvorens die ontwikkeling van PTST, deur die hiperadrenergiese reaksie onmiddelik na trauma-blootstelling, te inhibeer.

* * *

Die eerste doelwit van hierdie projek was om 'blootstelling aan roofdierreuk' (BRR) as 'n moontlike model van kliniese PTST te valideer en te bepaal of mannetjie Wistar-rotte verskillende vlakke van PTST-soortgelyke angste na blootstelling aan roofdierreuk sou demonstreer. Hiervoor het ons die uiterste gevalle van die normale verspreiding m.b.t. gedragsmerkers van angste geïdentifiseer. Dit gesê is die hoeveelheid fekale bolusse wat ten tye van die BRR-prosedure passeer is, gebruik as 'n maatstaf van onmiddelike trauma-geïnduseerde angste. Wat angsmetings 7 dae na BRR betref, het ons die verheue-

plusvormdoolhoftoets (VPT) gebruik om gedragskenmerke van kontrole en getraumatiseerde rotte te ondersoek. Hier is die getal besoeke aan die oop en toe arms onderskeidelik, sowel as die tyd wat rotte in elke arm spandeer het, gebruik as maatstawwe van angs-verwante gedrag. Verder het ons die aantal kopknikke wat van die oop arm af na die vloer van die gedragskamer gemaak is as 'n maatstaf van risiko-ontleding en verkenningsgedrag toegepas.

Oor die algemeen is die BRR-model van relevante etologiese waarde omdat dit natuurlike spanningsvolle gebeure naboots en as sulks langtermyn PTST-verwante gedrags-, neurochemiese-, en hormonale reaksies onttok. Die konsep waarop die model berus kan ook as geldig aanvaar word, omdat BRR 'n potensieel lewensbedreigende insident voorstel en daarom dien as 'n meer natuurlike spanningsneller vergeleke met ander vorme van prekliniese trauma, bv. elektriese stertskok of verstrengeling; laasgenoemde voorbeelde is eerder verwant aan martelingagtige ingrepe. Om die geldigheid van BRR as 'n model van PTST in ons eie laboratorium te ondersoek, het ons mannetjie Wistar-rotte gebruik; dit is immers vroeër reeds aangetoon dat Wistar-rotte uitermate sensitief vir spanning is. As sulks is Wistars uit verskillende eksperimentele groepe vir 10-minute lank onder dowwe wit lig (40 lux) aan 'n 10 cm x 10 cm-grootte materiaalblok blootgestel; sommige diere is blootgestel aan materiaalblokke wat nie met die reuk van 'n kat in aanraking gekom het nie (gedragskontroles), wyl die ander vir 2 maande aan 'n huiskat blootgestel is. Ten tye van die BRR-proses, sowel as gedurende die opvolgende VPT-analises, is die rotte se gedrag d.m.v. videomonitoring vasgelê en daarna geanaliseer met Ethovision XT® 13 sagteware, verkry van Noldus Inligtingstechnologie in Nederland. As 'n direkte gevolg van danige analises, bevestig data vooruitspruitend uit hierdie aspek van die studie dat BRR PTST-verwante angsiogeniese gedrag in ten minste 20 – 25% van die blootgestelde groep veroorsaak het; ons skryf die ander diere se nie-PTST-agtige gedrag toe aan voldoende salutogenese. Hierdie bevinding is belangrik omdat, soos voorheen genoem, trauma-blootgestelde individue nie almal op dieselfde manier na afloop van lewensbedreigende gevalle reageer nie. Die gevolgtrekking kan daarom gemaak word dat hierdie reaksie van BRR-blootgestelde diere, die geldigheid van die BRR-model t.o.v. sigwaarde, versterk.

* * *

Omdat 'n oorsaaklike rol vir abnormale hipotalamus-pituitêre aksisfunksionering in PTST bevestig is, het ons tweedens ten doel gehad om vas te stel of plasmakortikosteroonvlakke 21 dae nadat rotte aan skoon en kat-blootgestelde materiaalblokke onderskeidelik blootgestel

is, sou verskil. Verder wou ons vasstel of onmiddellike na-BRR, maar wel kroniese 21-dag-toediening van die selektiewe α_{2C} -adrenergiese reseptor (AR)-antagonis, ORM-10921, moontlike verskille in hierdie meting sou herstel. In hierdie verband het ORM-10921 nie juis noemenswaardige effek getoon nie, alhoewel dit genoem kan word dat die geneesmiddel 'n neiging tot hoër plasmakortikosteroonvlakke veroorsaak het. Verder was hierdie geringe toename postief gekorreleer met merkers van angsiolitiese gedrag.

* * *

Wat die rol van NA in die neurobiologie van PTST betref, het ons laastens ten doel gehad om vas te stel of ORM-10921 die moontlike gedragsversteurings wat deur BRR veroorsaak word, te verbeter. Wanneer die onbevredigende reaksie van PTST op bestaande farmakologiese terapieë, nl. selektiewe serotonienheropnameremmers, trisikliese antidepressante, sowel as propranolol, 'n β -AR-antagonis oorweeg word, bestaan daar duidelik 'n leemte betreffende die soeke na nuwe geneesmiddels wat vir die voorkoming en behandeling van PTST gebruik kan word. Dit is daarom van kliniese belang wanneer hierdie projek aantoon dat die toediening van ORM-10921 (0.3 mg/kg/dag) vanaf slegs 'n week na die BRR-prosedure (Aanhangsel C) vir 14 dae, angs-verwante gedrag versterk, eerder as verbeter. Interessant genoeg het ORM-10921 wat volgens hierdie skedule toegedien word, bygedra tot geringe stygings in die plasma-kortikosteroonkonsentrasies. Hierdie bevinding is ooreenstemmend met ons argument dat ORM-10921 wat eers vanaf 8 dae na BRR toegedien word, angiogenies mag wees.

Samevattend kan bevestig word dat hierdie studie die geldigheid van die BRR-model as 'n voorstelling van PTST-agtige gedrag op grond van sigwaarde, voorspelbaarheid, en konstrakte ooreenkomste, bevestig. Meer as dit selfs, het hierdie studie die rol van NA, meer spesifiek m.b.t. die rol van die α_{2C} AR in die patologie van PTST belig. Dit kan as sulks geargumenteer word dat die onmiddellike behandeling van trauma-blootgestelde individue 'n potensieel-voordelige effek in die voorkoming van PTST mag bemiddel.

Sleutelwoorde: PTST, blootstelling aan roofdierreuk (BRR), α_{2C} AR-antagonis, kortikosteroonkonsentrasie, katreuk, verhewe-plusvormdoolhoftoets (VPT)

CONGRESS PROCEEDINGS

Oral Presentation

- Investigating Predator Scent Exposure to Model Posttraumatic Stress Disorder Related Anxiety in Rats. Presented at the South African Society for Basic and Clinical Pharmacology Congress, 2-5 October 2017, University of the Free State, Bloemfontein, South Africa. The paper was awarded 3rd prize in the Young Scientist Competition, held under the auspices of the South African Society for Basic and Clinical Pharmacology.

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LIST OF ABBREVIATIONS

ACC	Anterior cingulate cortex
ACTH	Adrenocorticotrophic hormone
ADH -	Antidiuretic hormone
AMPA _-	Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AOB	Accessory olfactory bulb
APA	American Psychiatric Association
AR	Adrenoceptor
ASR	Acoustic Startle Response
AVP -	Arginine vasopressin
BCC	Behavioural cut-off criteria
BLA-	Basolateral amygdala
CA -	Cornus ammonis
cAMP	Cyclic adenosine monophosphate
CeA -	Central nucleus
CFR -	Condition fear response
CNS	Central nervous system
COMT	catechol-O-methyl transferase
CRF	Corticotrophin releasing factor
CRH-	Corticotrophin releasing hormone
DA-	Dopamine
DCS-	D-cycloserine
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
DOPAC	3-4-dihydroxyphenylacetic acid
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
ELS	Early-life stress

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EPM	Elevated Plus maze
FKBP5	FK506-binding protein 5
GABA	Amino-butyric acid
GAD	Generalized anxiety disorder
GR	Glucocorticoid receptor
GxE	Gene-environment
HD	Head dipping
HPA-	Hypothalamic-pituitary-adrenal
HVA	Homovanillic acid
KO	Knockout
KTCZ	Ketoconazole
LC	Locus coeruleus
IL-PFC	Infralimbic prefrontal cortex
IL-mPFC	Limbic medial prefrontal cortex
LTP	Long-term potentiation
IS-LH	Inescapable shock-learned helplessness
MAOIs	Monoamine oxidase inhibitors
m-CPP	Meta-chlorophenylpiperazine
mGluR 1-8	Metabotropic receptors
MR	Mineralocorticoid receptor
MOB	Main olfactory bulb
mPFC	Medial prefrontal cortex
mRNA	Messenger RNA
NA-	Noradrenalin
NE	Norepinephrine
NMDA	N-Methyl-D-aspartate
NOS	Nitric oxide synthase
NO	Nitric oxide

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NFK	Nuclear transcription factor
OAE	Open arm entries
OFT	Open Field Test
OE	Over-expression
PCP	Phencyclidine
PFC	Prefrontal cortex
PGi	Nucleus paragigantocellularis
PKA	Protein kinase
PSS	Predator Scent Stress
PSEM	Predator Scent Exposure Model
PTSD	Post traumatic stress disorder
PVN	Paraventricular nucleus
SG	Subcallosal gyrus
SNRIs	Serotonin norepinephrine reuptake inhibitors
SPS	Single prolonged stress
SSRIs	Selective serotonin reuptake inhibitors
TCA	Time in closed arms
TOA	Time in open arms
TCAs	Tricyclic antidepressants
TDS	Time-dependent sensitisation
TMT	2,3,5-Trimethyl-3-thiazoline
VNO	Vomerolateral organ
VTA	Ventral tegmental area
5-HT	Serotonin
5-HIAA	5-Hydroxyindoleacetic acid

I INTRODUCTION

1.1 Dissertation Layout

The current dissertation is compiled in the article format, as prescribed and approved by North-West University. As such, the main body of the dissertation is presented as a single manuscript that will be submitted to an international, peer reviewed neuroscience journal.

Chapter 1 provides a concise description of the project problem statement, study questions, aims, expected outcomes and a framework of the study layout. Chapter 2 comprises the literature background supporting the current project, while chapter 3 will present the key findings of the investigation in the form of a concept manuscript. This manuscript has been prepared according to the 'Instructions to Authors' as provided by the journal identified for submission (*viz. European Neuropsychopharmacology*) and will be presented as such. Chapter 4 summarizes the key findings of the project and concludes the study. The addendums contain a link to the 'Instructions to Authors' for *European Neuropsychopharmacology*, letters of permission of co-authors for subjecting the manuscript for examination purposes, and additional data generated throughout the course of this project that can be useful in future investigations.

Apart from the manuscript, the current dissertation has been prepared according to the referencing style of the American Psychological Association (APA), 6th ed. The dissertation is presented in UK English.

1.2 Problem Statement

PTSD is an anxiety disorder induced by exposure to a traumatic life-threatening event (Rauch & Foa, 2003; Uys *et al.* 2016). Consequently, PTSD has recently been classified by the American Psychiatric Association (APA) as a trauma and stress related disorder as opposed to its previous status as an anxiety disorder (American Psychiatric Association, 2013). The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) pays attention to the behavioural symptoms that accompany PTSD and proposes four distinct diagnostic clusters, instead of three as defined by the previous edition (DSM-IV). These are described as re-experiencing, avoidance, negative cognitions and mood, and arousal (American Psychiatric Association, 2013). However, the neurobiology of PTSD remains poorly understood while its pharmacological treatment is less than adequate (Hamner *et al.*, 2004). Furthermore, PTSD is associated with a diminished quality of life (Boscarino, 2004), high co-morbidity with other anxiety and mood disorders (Johnsen *et al.*, 2002) e.g. bipolar, depressive and general anxiety disorder, and an increased suicide-related mortality rate (Johnsen *et al.*, 2002). As such, it is anticipated that PTSD is set to become a major global health problem (Connor & Butterfield, 2003). The World Health Organization estimates that about 5 million deaths per year are caused by trauma and intentional and unintentional injuries. Almost 9 out of 10 (90%) of these injury-related deaths occur in low- and middle-income countries (LMICs), one of which is South Africa with a lifetime prevalence for PTSD in the general population being estimated at 2.3% (Swain, Pillay & Kliever *et al.*, 2017).

When considering the epidemiology of the illness, PTSD is observed in 15-50% of known trauma survivors and affects about 7% of the general population (Nemeroff *et al.*, 2006; Yehuda, 2009). Although trauma is necessary, it is not sufficient to induce PTSD. Furthermore, considering the variability in the prevalence and severity of PTSD (Milliken *et al.*, 2007), a critical question is “Why do some trauma victims develop PTSD whereas others that experience the same traumatic episode appear to be resilient?” (Davidson *et al.*, 2004).

With respect to the neurobiology underlying trauma-related adversity and stress, the noradrenergic system, in concert with corticotrophin releasing factor (CRF), plays an important role in conditioned fear responses and the retrieval of fear memory (Cahill, 2000). In fact, emotional events in patients with PTSD are associated with increased levels of NA in brain areas involved in learning and memory (McGaugh *et al.*, 2002; McGaugh, 2004), i.e. the amygdala and hippocampus, implicating a role for increased noradrenergic responses in PTSD.

Emotional memories are primarily facilitated by noradrenergic $\alpha_{1/2}$ and β_2 receptors (Cahill *et al.*, 1996), where emotionally aroused noradrenergic activation of the basolateral amygdala consolidates hippocampal contextual fear memory (Kim & Fanselow, 1992; Anagnostaras *et al.*, 1999). Furthermore, peripheral markers of a hyperadrenergic state in PTSD have also been found, e.g. high urine levels of NA (Yehuda *et al.*, 1992) and a decrease in the expression of platelet α_2 -receptors (Perry *et al.*, 1990). Together with findings demonstrating that adrenergic receptors in patients with chronic PTSD are hypersensitive (Southwick *et al.*, 1993), it is hypothesized that antiadrenergic agents relieve the symptoms of PTSD by blocking the increase in noradrenergic activity that is associated with fear and startle responses, ultimately resulting in curbing emotional arousal. The enhanced inhibitory effect that PTSD has on the HPA-axis causes patients with PTSD to have low cortisol levels despite having high CNS activity (Yehuda *et al.*, 1996; Baker *et al.*, 1999; Baker *et al.*, 2005). Of importance within the context of this study is that a suppressed cortisol response is associated with reduced stress-coping, thereby linking altered circadian cortisol release to the development of an anxiety and/or stress-related disorder (Dedovic & Ngiam, 2015).

Current pharmacological treatment regimens for PTSD include various classes of antidepressants, i.e. the selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs). Still, many patients remain refractory to treatment (Albucher & Liberzon, 2002; Ravindran & Stein, 2009) while the high rate and degree of comorbid major depression in many PTSD patients compromises a favourable treatment outcome (Ravindran & Stein, 2009). A number of seminal studies have noted the critical role of the noradrenergic system in the neurobiology of PTSD, both in humans (Hendrickson & Raskind, 2016) and animals (Harvey *et al.*, 2006). Previously, it has been reported that the $\beta_{1/2}$ adrenoceptor (AR) blocker, propranolol, and α_1 AR blocker, prazosin, are effective for certain PTSD-related manifestations, while stimulating the α_2 AR has been documented to worsen PTSD-related anxiety (Ruffolo Jr *et al.*, 1988; Starke, 2001) and to enhance fear memory (Kim & Fanselow, 1992; Anagnostaras *et al.*, 1999). Thus, given that immediate post-trauma propranolol administration has been shown to relieve amplified startle responses, nightmares and intrusive re-experiencing in some patients with PTSD (Friedman, 1997) while prazosin, administered 1 – 4mg/day post exposure, shows promise in treating sleep disturbances and nightmares in patients with chronic PTSD (Taylor & Raskind, 2002). Given the above evidence, there is increasing interest in the contribution and therapeutic potential of drugs that selectively

modulate the noradrenergic system in the treatment of PTSD. In this regard, alpha 2C ARs (α_{2C} ARs) regulate a diverse range of physiological processes, including sedation, vigilance, anxiety, pain and cardiovascular function (Ruffolo Jr *et al.*, 1988; Starke, 2001), while recent work has highlighted the potential of targeting these receptors for the treatment of mood, psychotic and cognitive disorders (Uys *et al.*, 2017). Indeed, PTSD is associated with co-morbid cognitive, mood and psychotic-like manifestations that are often problematic to treat (Johnsen *et al.*, 2002).

Work on non-selective alpha-2 antagonists (α_2) have shown that both α_{2A} and α_{2C} ARs inhibit the release of NA (Hein *et al.* 1999), although the α_{2C} -receptor system is less prominently involved in presynaptic inhibition than the α_{2A} -receptor (Bücheler *et al.* 2002). However, the potency of NA at the α_{2C} AR is higher than that at the α_{2A} AR, which also correlates with the difference in affinity of NA for the two receptors (Hein *et al.* 1999). Thus, NA inhibits its own release via the α_{2C} AR at lower endogenous NA concentrations compared to α_{2A} ARs (Bücheler *et al.*, 2002). Evidence from animal's studies with genetically altered α_{2C} AR expression and models predicting antipsychotic and antidepressant efficacy suggests that α_{2C} ARs play an important role in the modulation of monoamine neurotransmission in the brain, especially under stressful conditions (Lähdesmäki *et al.*, 2002). It has been found that the neurobiology of memory re-consolidation involves neurotransmitters like glutamate, noradrenalin and GABA (Uys *et al.*, 2017), while α_{2C} ARs modulate these transmitters (Uys *et al.*, 2017). These qualities are of importance especially when considering selective α_{2C} AR ligands in animal models of human disease characterised by hyperadrenergic states, such as PTSD (Yamamoto, Hornykiewicz 2004), and explains why noradrenergic blockade may be useful in attenuating traumatic memories in PTSD, even well-consolidated old memories (Debiec & LeDoux, 2006). Moreover, the non-selective and α_{2A} ARs preferring agonists clonidine and guanfacine have been reported to ameliorate and the selective α_2 AR antagonist atipamezole to worsen the Phencyclidine (PCP) induced visual spatial and working memory deficit (Debiec & LeDoux, 2006). Taken together, these experiments emphasize the possible beneficial effect of a subtype selective α_{2C} AR compound (ORM-10921) on cognitive function and that the effects are different from the effects produced with non-selective α_2 AR drugs. Studies by Sallinen and colleagues (Sallinen *et al.*, 2013) and Uys and colleagues (2016; 2017) confirm the beneficial effects of α_{2C} AR antagonist, in mood and psychosis-related disorders, while also demonstrating the role of α_{2C} AR antagonism in the alleviation of hypoglutaminergic

states including social and cognitive dysfunctions (Sallinen *et al.*, 2013). Importantly, several studies have reported opposing roles of α_{2C} vs. α_{2A} AR antagonists with respect to mood and cognitive parameters, highlighting that non-selective α_2 AR antagonism may be counter-productive (Uys *et al.*, 2017). Further, considering stress-related conditions, animal studies have demonstrated that hippocampal over expression of α_{2C} ARs (α_{2C} -OE) increases immobility (depressive-like behaviour) in the forced swim test while being associated with increased corticosterone levels following repeated stress (Sallinen *et al.*, 1999; Uys *et al.*, 2017). The opposite is demonstrated under conditions of α_{2C} -receptor knockout (KO), presenting with antidepressant effects (Sallinen *et al.*, 1999; Uys *et al.*, 2017). With respect to monoamines, α_{2C} -KO mice present with reduced serotonin (5HT), 5-hydroxyindoleacetic acid (5HIAA), 3-4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) compared to α_{2C} -OE subjects (Sallinen *et al.*, 1999).

Suitable animal models of PTSD are critical to study the mechanisms underlying trauma-induced changes, to investigate the physiological and neurobiological aetiologies underlying PTSD, as well as to test novel drug treatments (Stam, 2007). In an attempt to imitate a psychiatric disorder in a laboratory animal, three principles of validity need to be met, *viz.* face, construct, and predictive validity (Overstreet, 1993). Collectively these criteria contribute to the strength of an animal model and ensure that findings from investigations in valid animal models are useful and meaningful (Bird & Parlee, 2000).

Considering the current investigation, the predator scent exposure (PSE) model has face validity as it involves an intense traumatic experience and has previously been reported to result in long term changes in behavioural, autonomic and hormonal responses in rats that correlate with symptoms seen in humans with PTSD (Cohen *et al.*, 2003). Regarding the conceptualization of the model, 'predator exposure trauma' resembles a potentially life-threatening situation and may emulate a more "likely and natural" challenge than other forms of stressors, e.g. electric tail shocks or physical restraint (Adamec *et al.*, 1997). With respect to symptomological similarity between the human condition and the animal, the PSE model has been shown to elicit hyperarousal (Cohen *et al.*, 2004; Cohen *et al.*, 2006; Lewitus *et al.*, 2008; Cohen *et al.*, 2009), increased levels of anxiety (Adamec *et al.*, 2006; Muñoz-Abellán *et al.*, 2008), social withdrawal (Zangrossi & File, 1992), and freezing and avoidance behaviour (Blanchard, 1990; Wallace & Rosen, 2000; Dielenberg & McGregor, 2001; Masini *et al.*, 2005), all consistent with clinical manifestations in humans with PTSD. Furthermore, regarding the

purported molecular and biological similarities to PTSD, changes evident in the animal model correlate with that described in the clinical condition and contribute to its construct validity. Indeed, the PSE model has proved to be a valuable framework to study HPA-axis abnormalities relevant to PTSD (Yehuda & Antelman, 1993) as it mimics the clinical neurochemical and neuroendocrine changes of the human condition, e.g. HPA-abnormalities, increased sympathetic activity, increased adrenocorticotrophic hormone (ACTH) concentrations, diminished vagal tone and a shift in the sympathovagal balance (Cohen *et al.*, 2003), all consistent with clinical findings in PTSD. Moreover, animals exposed to predator scent also display an enhanced sensitivity to negative glucocorticoid feedback that is often characteristic of PTSD and leads to hypocortisolemia (Cohen *et al.*, 2003). Pertaining to the predictive validity of the model, noradrenergic receptor antagonists, e.g. propranolol (Lennartz *et al.*, 1996; Ferry *et al.*, 1999; Pitman *et al.*, 2002) steroid synthesis inhibitors, e.g. ketoconazole (KTZ; Cohen *et al.* 2000), chronic antidepressant treatment, as well as alcohol (Blanchard *et al.*, 1990; Blanchard *et al.*, 1993), attenuate stress-related behaviours in this model, as has been demonstrated in clinical PTSD (Blanchard *et al.*, 1990).

Given that PTSD affects only 15 – 50% of trauma-exposed individuals (Cohen *et al.*, 2005b; Yehuda, 2009), Yehuda and Antelman (1993) proposed that individual variation in and susceptibility to trauma-related pathology must be a key criterion for an animal model of PTSD. Therefore, the study population should be defined in congruence with clinical PTSD studies (Cohen *et al.*, 2003) and thus focus specifically on afflicted animals, i.e. those that have developed marked anxiety and fear-related behaviour following trauma exposure, in comparison to the apparently resilient individuals. Moreover, data analyses from animal studies should be done in a manner that reflects as closely as possible the DSM-5 criteria (Cohen *et al.* 2003) and as such, the concept of ‘defining and categorizing the afflicted’ has received much attention in pre-clinical investigations (Cohen *et al.*, 2003; Cohen *et al.*, 2004; Cohen *et al.*, 2005b). Applying so-called behavioural cut-off criteria (BCC) has important implications for the face, construct, and predictive validity of preclinical studies of PTSD (Cohen *et al.*, 2003; Cohen *et al.*, 2004; Cohen *et al.*, 2005b). Briefly, rats are divided into well adapted and maladapted groups, with maladapted rats presenting with the neurobiological and neuroendocrine changes highlighted earlier, e.g. increased sympathetic activity, diminished vagal tone and an increase in the sympathovagal balance (Cohen *et al.*, 2003; Cohen *et al.*, 2004; Cohen *et al.*, 2005b). Such animals also exhibit higher mean startle responses than their well-adapted controls. BCC therefore identifies a valid sample of stress sensitive animals. In

the present investigation, apart from employing BCC, with some modification, male Wistar rats will be employed due to their demonstration of stronger conditioned and unconditioned responses to stress when compared to other strains, e.g. Sprague-Dawley rats (Staples & McGregor, 2006).

Drawing from the above, we hypothesize that selectively modulating the noradrenergic system may represent an important neurobiological target in the treatment of PTSD. As explained earlier, various clinical (Strawn & Geraciotti, 2008) and preclinical (Bryant *et al.*, 2009; Holbrook *et al.*, 2010) studies have revealed the potential of immediately targeting noradrenergic receptors following trauma exposure, especially antagonizing β_1 - and α_1 receptors. However, the effects of targeting the α_{2C} AR in PTSD remains unexplored, mainly due to the fact that ligands for the α_{2C} AR subtype have, until recently, been unavailable (Uys *et al.*, 2017). This constitutes the departure point for the present study, in which the role of the α_{2C} -receptor in the neurobiology and treatment of PTSD will be addressed. As such, this study will first set out to validate the predator-induced trauma model of PTSD, viz. PSE, in male Wistar rats in order to provide a valid research platform for the investigation. Thereafter and considering literature that suggests that the $\beta_{1/2}$ AR antagonist, propranolol, may have clinical utility in preventing the development of PTSD when administered immediately post-trauma, we will investigate the effects of chronic administration of the selective α_{2C} AR antagonist, ORM-10921, initiated immediately and continuing for 21 days following exposure to either predator (domestic cat) scent or scent-free cloth on anxiety-related behaviours and corticosterone levels; we will further compare these to findings in respective drug-naïve control cohorts. In addition, in order to confirm the supposed superior efficacy of noradrenergic interference immediately post-trauma as opposed to later, we have investigated the effects of chronic administration of the selective α_{2C} AR antagonist, ORM-10921, initiated on day 8 post exposure to either predator (domestic cat) scent or scent-free cloth on anxiety-related behaviours and corticosterone levels and compare these to findings in respective drug-naïve control cohorts and immediate treatment, to determine the time frame at which drug should be administered.

1.3 Study Questions

The current study is designed to systematically address the face, construct, and predictive validities of the Predator Scent Exposure Model (PSEM), and is conceptualized to re-examine the diverse and complex role of noradrenergic receptors in PTSD. Therefore, the following study questions are asked:

Study Question	Applicable literature
• <u>Manuscript A (Chapter 3)</u> As is true for the human population, will male Wistar rats demonstrate different levels of anxiety akin to maladaptation and well-adaptation following exposure to a traumatic event, i.e. predator scent, as observed in the elevated plus maze (EPM)?	(Cohen <i>et al.</i> , 2003; Sallinen <i>et al.</i> , 2007; Sallinen <i>et al.</i> , 2013; Uys <i>et al.</i> , 2017)
• <u>Manuscript A (Chapter 3)</u> Given evidence indicating altered plasma cortisol in patients with PTSD, will altered corticosterone concentrations be demonstrated in animals exhibiting PTSD-like behaviour and will such alterations be sensitive to chronic treatment with ORM 10921, as opposed to treatment with a vehicle control?	(Cohen <i>et al.</i> , 2003; Cohen <i>et al.</i> , 2005b; Yehuda, 2009)
• <u>Manuscript A (Chapter 3)</u> Based on the role of a hyperadrenergic state in the manifestation of PTSD, and considering the less than adequate response of clinical PTSD to modulation of the noradrenergic and serotonergic systems, i.e. chronic treatment with <i>inter alia</i> TCAs, SNRIs, and SSRIs, as well as acute treatment with clonidine, will selective antagonism of the α_{2C} AR with the novel compound, ORM-10921, reverse the above-mentioned biobehavioural changes induced by PSEM ?	(Yamamoto & Hornykiewicz, 2004)
• <u>Manuscript A and Supplementary Data (Chapter 3 and Addendum C)</u> Based on clinical controversy pertaining to the time post-trauma when treatment should be administered, will ORM-10921 administered immediately vs. one week following trauma respectively, elicit differences in how it addresses PSEM-induced bio-behavioural changes?	(Cohen <i>et al.</i> , 2000; Cohen <i>et al.</i> , 2006)

1.4 Project Aims

To address study question one (data presented in Chapter 3), we aimed to:

- Validate the predator scent exposure model in our laboratory and separate animals into well-adapted and maladapted cohorts based on performance in the EPM;

To address study question two (data presented in Chapter 3 and Addendum C), we aimed to:

- Compare plasmacorticosterone concentrations between treatment-naive well-adapted and maladapted animals;

To determine the outcome of study question three (data presented in both Chapter 3 and Addendum C), we aimed to:

- Assess the bio-behavioural (brain and physiological function) responses of well adapted and maladapted trauma-exposed animals to chronic subcutaneous administration of ORM-10921 (0.3 mg/kg/day), administered immediately (21-day treatment from day 0 until day 21) or from day 8 (14-day treatment from day 8 until day 21) post trauma exposure and compare such changes to that observed in the respective vehicle treated control groups;

To determine the outcome of study question four (data presented in both Chapter 3 and Addendum C), we:

- Assessed the bio-behavioural responses of well-adapted and maladapted animals to chronic subcutaneous treatment with ORM-10921 (0.3 mg/kg/day), beginning immediately (21-day treatment from day 0 until day 21) or from day 8 (14-day treatment from day 8 until day 21) post trauma exposure.

1.5 Project layout

The sequence of events on day 1 – 22 for all rats:

- Male Wistar rats (n = 160) were exposed individually under dim white light (15 lux) for 10 min to either a 10 x 10 cm predator scent free cloth (n = 55) or a cloth that has been exposed to a male cat (cat cloth) for 2 months (n = 105) (Cohen *et al* 2003; Cohen *et al.* 2005, Yehuda 2009) while being video recorded.
- The soiled or control cloths (n = 160) have been introduced into the outer corner of a rodent holding cage, whereupon each rat could freely explore the cage for a period of 10 min.
- At the end of the exposure period, rats have been removed and placed into the normal holding cages where they have been housed in groups of 4 – 5.
- Cages were cleaned between trials to remove any trail of the previous rat and a new cloth (10 cm X 10 cm) was introduced. This was done to prevent confounding effects of previous rat and predator scent on newly introduced subjects.
- To validate PSEM as a potential model of PTSD, each rat in Groups I and II (see below) was subjected to the EPM 7 days following PSE to measure individual anxiety-like manifestations and characterize PTSD-like behaviour.
- For Groups III – VI (see below), treatment has been initiated either 1 hour following PSEM and maintained for 21 days (data presented in Chapter 3), or from day 8 following PSEM and maintained for 14 days (data presented in Addendum C).
- For animals in Groups III – VI (see below), behavioural assessment in the EPM were repeated following the full course of drug administration, i.e. on day 21. On the next day (day 23), rats were decapitated, and trunk blood was collected and stored until the date of bioanalysis.

To establish PSEM as a potential model of PTSD, the well-adapted and maladapted cohorts (Groups I and II; see below) were categorized as following:

Well-Adapted

Based on column analyses of data generated in the EPM, well-adapted animals were defined as those individuals clustered within the upper quartile of the normal distribution relating to the number of entries into and time spent in the open arms (i.e. the extreme of less anxious behaviour), and/or being clustered within the lower quartile of the normal distribution relating to the number of entries into and time spent in the closed arms (i.e. the extreme of less anxious behaviour).

Maladapted

- Based on column analyses of data generated in the EPM, maladapted animals are defined as those individuals clustered within the lower quartile of the normal distribution relating to the number of entries into and time spent in the open arms (i.e. the extreme of high anxiety behaviour), and/or being clustered within the upper quartile of the normal distribution relating to the number of entries into and time spent in the closed arms (i.e. the extreme of high anxiety behaviour).

1.6 Study Design and Animal Groups

To address all four of the study questions alluded to above, the following groups have been broadly constituted from the larger pool of animals:

Establishing PSE as a potential model of PTSD:

- Group 1 - Clean cloth control group measuring response in the EPM (n = 31); also used in the treatment study initialized on day 8 following PSE;
- Group 2 - Cat cloth group measuring response in the EPM (n = 31); also used in the treatment study initialized on day 8 following PSE

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Determination of treatment response and elucidating the most appropriate time post-trauma exposure for therapeutic intervention (see Figure 1):

- Group III - non-scented (n = 8) exposed vehicle treated control group, with treatment initiated immediately following cloth exposure and continuing for 21 days;
- Group IV - cat cloth (n = 12) exposed vehicle treated control group, with treatment initiated immediately following cloth exposure and continuing for 21 days;
- Group V - non-scented (n = 16) exposed ORM-10921 (0.3 mg/kg/day) treated group with treatment initiated immediately following cloth exposure and continuing for 21 days;
- Group VI - cat cloth (n = 24) exposed ORM-10921 (0.3 mg/kg/day) treated group with treatment initiated immediately following cloth exposure and continuing for 21 days;
- Groups VII and VIII - non-scented (n = 12 from Group I) and cat cloth (n = 37; 13 from Group II + Group VIII 24 animals) exposed vehicle treated control groups, with treatment initiated on day 8 following exposure and continuing for 14 days only; and
- Groups IX and X - non-scented (n = 19 from Group I) and cat cloth (n = 32; 18 from Group II + Group X 14 animals) exposed ORM-10921 (0.3 mg/kg/day) treated groups with treatment initiated on day 8 following exposure and continuing for 14 days only.

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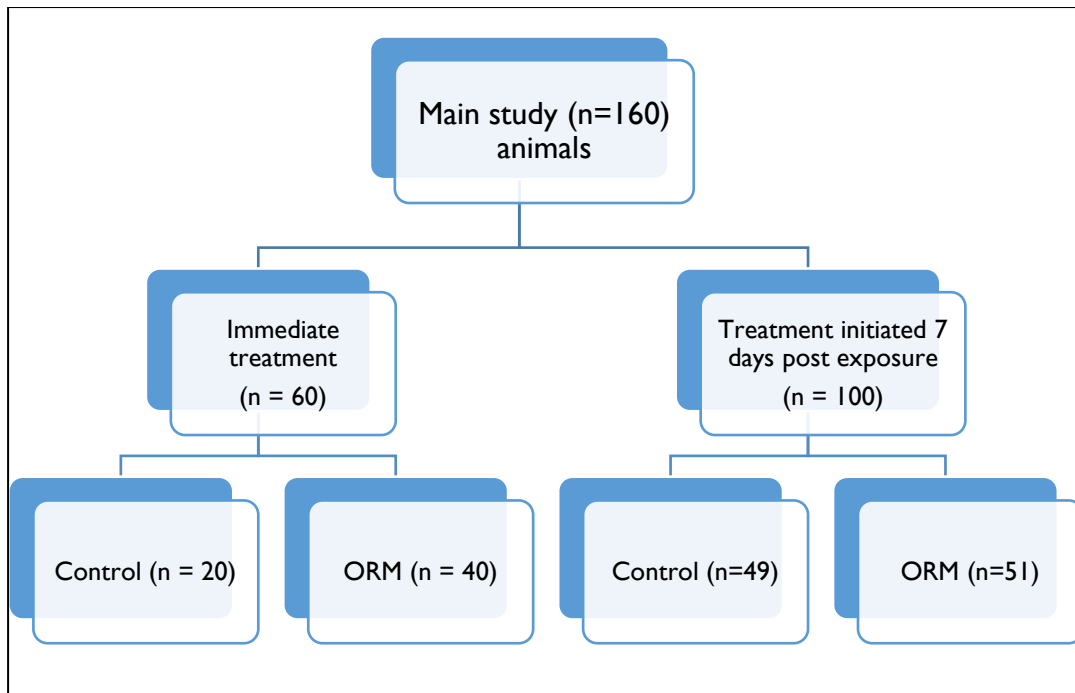


Figure I-1 - Animal group description

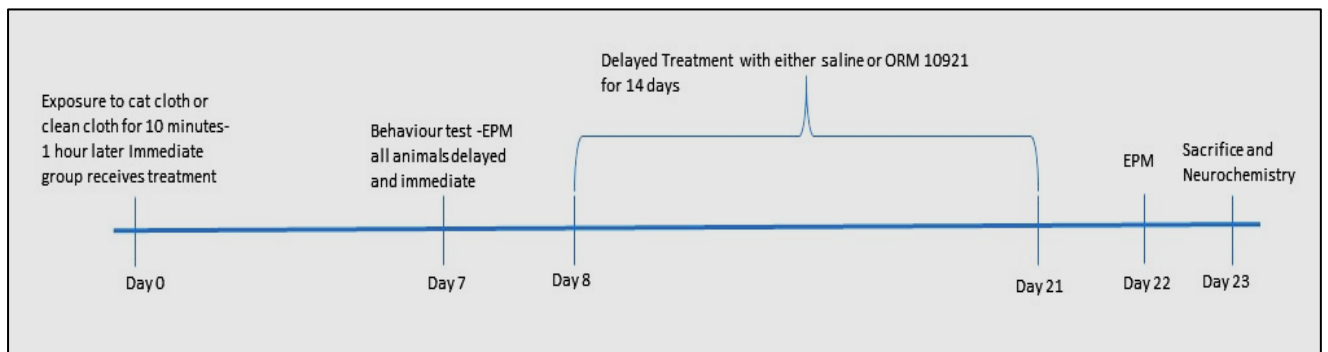


Figure I-2 - Time line of study of study design

1.7 Expected Results

Study Question	Expected outcome
1) Can male Wistar rats be clustered into well-adapted and maladapted cohorts following predator scent exposure?	It is expected that at least 20 – 25% of the total group of individuals will develop anxiety-like behaviours as identified by data obtained from correlation analyses of the normal distribution, while the remainder of the group will remain resilient.
2) Will maladapted animals present with altered post-trauma plasma corticosterone concentrations compared to well-adapted individuals?	It is expected that PSE will result in PTSD-like changes in plasma corticosterone concentrations (Cohen <i>et al.</i> , 2003; Cohen <i>et al.</i> , 2005a; Cohen <i>et al.</i> , 2006; De Kloet <i>et al.</i> , 2008) in the maladapted, but not well-adapted clusters.
3) Will selective antagonism of the α_{2C} AR antagonist ORM-10921 result in reversal of any observed bio-behavioural responses as noted in study questions 2 and 3?	Based on interplay between the effects of the α_{2C} adrenoceptor and corticosterone in the manifestation of anxiety and fear memory consolidation (Cahill, 2000), it is expected that the selective antagonism of said receptor with ORM-10921 will elicit a robust and significant improvement in maladapted bio-behavioural manifestations in maladapted individuals compared to those maladapted animals treated with the vehicle alone (Cohen <i>et al.</i> , 2003; Cohen <i>et al.</i> , 2004; Cohen <i>et al.</i> , 2005a)
4) Will the time of administration of ORM-10921 play a role in treatment outcome?	Based on the time frame in which treatment was administered (1 hour vs. 7 days after stress exposure), it is expected that the time of administration of ORM-10921 conforms to the time frame within which the memory consolidation process takes place at the cellular level, and that ORM 10921 will be more effective in preventing the above-mentioned PSE-mediated bio-behavioural changes when treatment is initiated immediately after stress exposure as opposed to 7 days later (Cohen <i>et al.</i> , 2006)

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2 LITERATURE REVIEW

2.1 PTSD in the Clinical Environment

Posttraumatic stress disorder (PTSD), is a psychiatric disorder that can manifest following the experience or witnessing of a life-threatening event such as military combat, natural disasters, terrorist incidents, serious accidents, or physical or sexual assault during child- or adulthood (American Psychiatric Association, 2013). Although most survivors of a traumatic event recover over time, approximately 30% of victims will go on to develop full-blown PTSD (Nemeroff *et al.*, 2006). PTSD was first conceptualized as a diagnosis in 1980 and is categorised as a trauma and stress related disorder in the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013; Association, 2013). The socio-economic impact of PTSD is significant as evinced by poor social and family relationships, absenteeism from work, lower income, and lower educational and occupational success (American Psychiatric Association, 2013; Association, 2013).

2.1.1 The symptoms and diagnosis of PTSD

PTSD can occur at any age, beginning after the first year of life. Symptoms of PTSD usually begin within the first 3 months after exposure to the traumatic event, although there may be a delay of months, or even years, before criteria for its diagnosis can be applied (American Psychiatric Association, 2013).

The clinical presentation of PTSD varies. In some individuals, fear-based re-experience as well as emotional and behavioural symptoms may be dominant. In others, anhedonic or dysphoric mood states and negative cognitions may be most distressing. Further, individuals may present with arousal and reactive-externalizing symptoms, while dissociative symptoms often predominate. However, individuals may also exhibit combinations of these symptom patterns (American Psychiatric Association, 2013).

The DSM-V pays attention to the behavioural symptoms that accompany PTSD and proposes four, instead of three (DSM-IV) distinct diagnostic clusters (American Psychiatric Association, 2013). They are described as re-experiencing, avoidance, negative cognitions and mood, and arousal (American Psychiatric Association, 2013). Re-experiencing can be described as spontaneous intrusive memories of the traumatic event, as well as recurrent dreams,

flashbacks or other related experiences of intense or prolonged psychological distress (American Psychiatric Association, 2013). These memories can cause both emotional and physical reactions linked to the previous experience of the actual event. Avoidance refers to attempts to evade any stimuli that may link to the traumatic event, e.g. distressing memories, thoughts, feelings or external physical reminders of the event (American Psychiatric Association, 2013), while patients must recognise that the event is no longer occurring. However, in PTSD, general declarative memory, which refers to memories that are consciously recalled, viz. facts and verbal knowledge, as well as explicit information about the trauma, is compromised while non-declarative memory, i.e. long-term memory that does not require conscious thought, is bolstered (Elzinga & Bremner, 2002; Buwalda *et al.*, 2005). Therefore, although a PTSD patient may recognise that the actual traumatic event no longer occurs, these alterations in memory processing contribute to the continuous re-experiencing of trauma, ultimately resulting in excessive avoidance. Negative cognitions and mood represents a myriad of feelings ranging from a persistent and distorted sense of blame of self or others, estrangement from others, markedly diminished interest in activities and an inability to remember key aspects of the event (American Psychiatric Association, 2013). Arousal is marked by aggressive, reckless or self-destructive behaviour, sleep disturbances, hypervigilance or related problems (American Psychiatric Association, 2013). In fact, one of the major symptoms in PTSD is the low threshold for environmental stimuli to elicit arousal of exaggerated affective responses such as fear and anxiety (Yehuda, 2006).

Further, as many conditions share some symptomology with PTSD, e.g. generalized anxiety disorder (GAD) and phobia (DSM-V), clear diagnostic criteria for PTSD have been defined by the DSM-V (American Psychiatric Association, 2013). These are reproduced as follows:

Criterion A: Stressor

- Where the individual experiences the traumatic event(s) directly or witnesses the event(s) in person, or as it occurred to others and that involves actual or threatened death or serious injury, or a threat to the physical integrity of oneself or others;

- Where the individual learns about the traumatic event as they occurred to a close family member or close friend. In cases of actual or threatened death of a family member or friend, the event must have been violent or accidental; or
- Where the individual experiences repeated or extreme exposure to aversive details of the traumatic event.

Criterion B: Intrusive recollection

Presence of intrusion symptoms associated with the traumatic event, beginning after the traumatic event:

- Recurrent, involuntary, and intrusive distressing memories of the traumatic event. Note: in young children, mainly older than 6 years, repetitive play may occur in which themes of the traumatic event are expressed;
- Recurrent distressing dreams that is related to the traumatic event. Note: in children, there may be frightening dreams without recognizable content;
- Dissociative reactions (e.g. flashbacks) in which the individual feels or acts as if the traumatic event was actually happening (which includes a sense of reliving the experience, illusions, hallucinations, including those that occur upon awakening or when intoxicated). Note: in children, trauma-specific re-enactment may occur in play.
- Intense or prolonged psychological distress at exposure as well as marked physiological reactions to internal or external cues that symbolize or resemble an aspect of the traumatic event.

Criterion C: Avoidance

Persistent avoidance of stimuli associated with the traumatic event, as evidenced by one or both of the following:

- Avoidance of the memories of the event or efforts to avoid thoughts or feelings about the traumatic event;
- Avoidance of external reminders such as people, places, conversations, activities, objects, situations that cause distressing memories about the trauma.

Criterion D: Negative cognition and mood

Negative alterations in cognitions and mood associated with the traumatic event, worsening after the traumatic event has occurred, as evidenced by two (or more) of the following:

- Inability to recall an important aspect of the trauma (typically due to dissociative amnesia and not to other factors such as head injury, alcohol, or drugs);
- Persistent and exaggerated negative beliefs about oneself, others, or the world;
- Distorted cognitions about the cause of the traumatic event that leads the individual to blame himself/herself or others;
- Persistent negative emotional state (e.g., fear, horror, anger, guilt, or shame);
- Markedly diminished interest in significant activities that once interested the person;
- Feelings of detachment or estrangement from others;
- Persistent inability to experience positive emotions (unable to have loving feelings)

Criterion E: Hyperarousal

Marked alterations in arousal and reactivity associated with the traumatic event, worsening after the traumatic event has occurred, as evidenced by two (or more) of the following:

- Irritable behaviour and angry outbursts which are typically expressed as verbal or physical aggression toward people or objects;
- Reckless or self-destructive behaviour;
- Hypervigilance;
- Exaggerated startle response;
- Problems with concentration;
- Difficulty falling or staying asleep or restless sleep

Criterion F: Duration

- Duration of the disturbance (Criteria B, C, D, and E) is more than 1 month.

Criterion G: Functional Significance

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

Criterion H: Disturbance

- The disturbance is not attributable to the physiological effects of a substance (e.g. medication, alcohol) or another medical condition.

2.1.2 Epidemiology and comorbidity and impact on the quality of life

PTSD is observed in 15 – 50% of known trauma survivors and affects about 7% of the population (Nemeroff *et al.*, 2006). In the United States, combat exposure has been reported to account for about 27.8% of PTSD diagnosed in men, while almost half of combat veterans have experienced ‘clinically serious stress reaction symptoms’. Further, clinical studies (Yehuda, 2009) have reported that 15 – 25% of individuals exposed to a traumatic experience go on to develop PTSD. Trauma is necessary but not sufficient for developing PTSD. In fact, one of the most critical questions at this time is why only some trauma victims develop PTSD (Davidson *et al.*, 2004) whereas others experiencing the same trauma appear to be resilient. In addition, those who meet the criteria for PTSD vary widely with respect to symptom severity and symptom phenomenology (Dickie *et al.*, 2008). A variety of factors contribute to the magnitude of PTSD symptoms, including genetic predisposition, social support network and early life experiences (Jovanovic & Ressler, 2010). In other words, there are distinct genetic, environmental and psychosocial factors that determine an individual’s resilience to trauma.

The main causes of PTSD in South Africa are events such as criminal victimization, car accidents as well as abuse seen in children, e.g. sexual, physical or emotional abuse (Nemeroff *et al.*, 2006). Women have twice as high risk for developing PTSD symptoms than men even if exposed to the same form of trauma, while their symptoms are often more persistent (Holbrook *et al.*, 2002; Van Loey & Van Son, 2003). Importantly, males and females respond differently to psychological stress, possibly relating to hormonal differences (Ter Horst *et al.*, 2012). It has been argued that the increased risk for PTSD in females is attributable to a greater likelihood of exposure to traumatic events such as rape and other forms of interpersonal violence (American Psychiatric Association, 2013).

Considering comorbidity, individuals with PTSD are 80% more likely than individuals without PTSD to present with symptoms of another mental disorder, e.g. depression, bipolar disorder, anxiety or substance use disorder. Importantly, such comorbid disorders, such as depression, are invariably highly treatment resistant (Johnsen *et al.*, 2002). Although most young children

with PTSD may have at least one other diagnosis, the patterns of comorbidity are different than in adults, with oppositional defiant disorder and separation anxiety disorder being more predominant (Molnar *et al.*, 2001). Finally, there is considerable comorbidity between PTSD and major neurocognitive disorders with overlapping symptoms, anxiety, aggression, and irritability (American Psychiatric Association, 2013).

2.1.3 Genetic implications of PTSD

The brain functions as the main organ facilitating stress responses due to its ability to perceive and analyse life threatening scenarios and to trigger the physiological and behavioural responses to severe stressors (McEwen & Gianaros, 2011). Furthermore, it can adapt and change with each stressful experience. As such, the brain undergoes numerous structural changes, i.e. neuronal replacement, dendritic remodelling and higher rates of synapse turnover when responding to the environment (McEwen & Gianaros, 2011). Stress causes an imbalance of the neural circuitry underlying cognition, anxiety, mood, and decision making (McEwen & Gianaros, 2010). In the event of short-term exposure to stress, said changes may serve a protective role and prevent further manifestations of psychiatric symptomology. However, if the danger passes but the trauma-related behavioural state persists along with neural maladaptation, a combination of pharmacological and behavioural therapy may be necessary to modify adverse neuropsychiatric outcomes (McEwen & Gianaros, 2011). Moreover, as noted earlier, the extent of such adaptation is dependent on a number of factors. In this paragraph, we will focus on the genetic construct of PTSD.

2.1.4 Early life adversity

It has been found that early life stress (ELS) tends to increase the risk for both adult traumatisation and PTSD (Cogle *et al.*, 2010) following exposure to a traumatic event (Brewin *et al.*, 2000). Therefore, if the risk for developing PTSD following a traumatic event is linked to underlying genetic vulnerability, it would be expected that biological relatives of the individual with PTSD would also have a higher risk to developing the condition compared to non-relatives (Skelton *et al.*, 2012). Indeed, it was found that PTSD diagnoses were more frequent in adult children of holocaust survivors with PTSD compared to those survivors devoid of psychiatric symptoms (Yehuda *et al.*, 2001). Further, much of the research into the genetic basis for PTSD has been gathered via twin studies and has indicated that heritability accounts for 30 – 40% of the variance in risk for PTSD (True *et al.*, 1993; Stein *et al.*, 2002).

These studies have revealed complex interactions between genetics and environmental factors and have shown that specific genetic polymorphisms are mostly found in the regulatory promotor and not the coding regions of genes (Stein *et al.*, 2008). As only approximately 30% of individuals who survive a traumatic or life-threatening event will develop PTSD (Nemeroff *et al.*, 2006), it would be valuable if vulnerable individuals can be identified as soon as possible following trauma exposure with appropriate interventions introduced to prevent the subsequent development of PTSD. Essential to this approach is to bolster the resilience of predisposed patients (Jovanovic & Ressler, 2010). Resilience, being an adaptive response that maintains homeostasis under stressful circumstances (Jovanovic & Ressler, 2010), can be compromised by traumatic stress resulting in an increased vulnerability to develop psychiatric illness (Jovanovic & Ressler, 2010). As various factors can contribute to resilience, including neurobiology and psychological profile as well as environmental impact (Charney, 2004), it has been proposed that the level of resilience displayed results from a combination of inheritable and environmental factors (Figure 2-1).

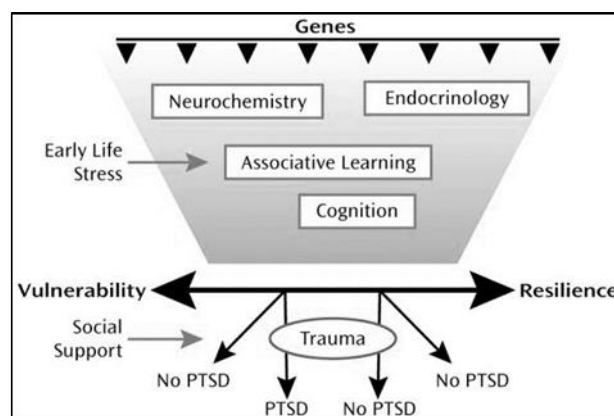


Figure 2-1 - Schematic diagram of genetic, neurobiological, and environmental interactions that contribute to vulnerability or resilience in relation to PTSD (Jovanovic & Ressler *et al.*, 2010)

The biological factors can be linked to inheritable genetic profiles that code for specific neurochemical and neuropsychological constructs that have been found to promote resiliency (Charney, 2004). Furthermore, said genetic profiles may also code for associative learning mechanisms, i.e. fear conditioning in response to enhanced fear responses or that promote fear extinction of previously fearful stimuli (Charney, 2004).

2.1.5 Gene-environment interactions in PTSD

Since PTSD by definition requires exposure to a traumatic environmental event (Criterion A) and considering that individuals vary in their phenotypic response to such events, the genetic basis of PTSD can be explained by studies of gene-environment (GxE) interactions, rather than focusing on the main effects of genes alone (Hubler & Scammell, 2004). Indeed, recent studies (Hubler & Scammell, 2004), have demonstrated interactions between FK506-binding protein 5 (FKBP5) gene polymorphisms and exposure to traumatic events during childhood in predicting the severity of PTSD (Hubler & Scammell, 2004). FKBP51/FKBP5 is a 51-kDa immunophilin that belongs to the family of FK506-binding proteins, originally named after their ability to bind the immunosuppressant FK506 (Wiederrecht *et al.*, 1992). Also, FKBP5, a gene encoding a co-chaperone protein that interacts with heat-shock protein 90 (hsp90), reduces glucocorticoid receptor (GR) translocation and sensitivity (Hubler & Scammell, 2004). Briefly, FKBP5 is upregulated following prolonged GR activation, while polymorphisms associated with heightened FKBP5 expression can provoke increased GR resistance and a decreased negative feedback response. This leads to prolongation of the stress response following exposure to a traumatic event.

To understand how the above-described effects it is important to briefly review the function of the primary effector of the stress response, the hypothalamic–pituitary–adrenal (HPA) axis (see section 2.2.1.). As depicted in Figure 2-2, glucocorticoids move in to the cytoplasm (a) and activate the GR complex. FKBP5 binding to the complex decreases the affinity of glucocorticoids to the GR and delays translocation of the GR to the nucleus. The exchange of FKBP5 for FKBP4 (b) results in GR translocation to the nucleus (c). The GR can either interact as a monomer with other transcription factors (d) or form a homodimer that binds to deoxyribonucleic acid (DNA) at glucocorticoid response elements. Overall, GR functions result in transactivation or transrepression of many genes. The FKBP5 gene is highly responsive to GR, but responsiveness depends on FKBP5 polymorphisms and methylation status (e). The synthesized FKBP5 mRNA translocate to the cytoplasm (f) where it is translated into FKBP5 protein. FKBP5 then inhibits GR activity not only forming an ultra-short, intracellular negative feedback loop of GR signalling but also modulating several other biological pathways (g) (Zannas *et al.*, 2016). The role of FKBP5 in GR signalling is summarized in Figure 2-2.

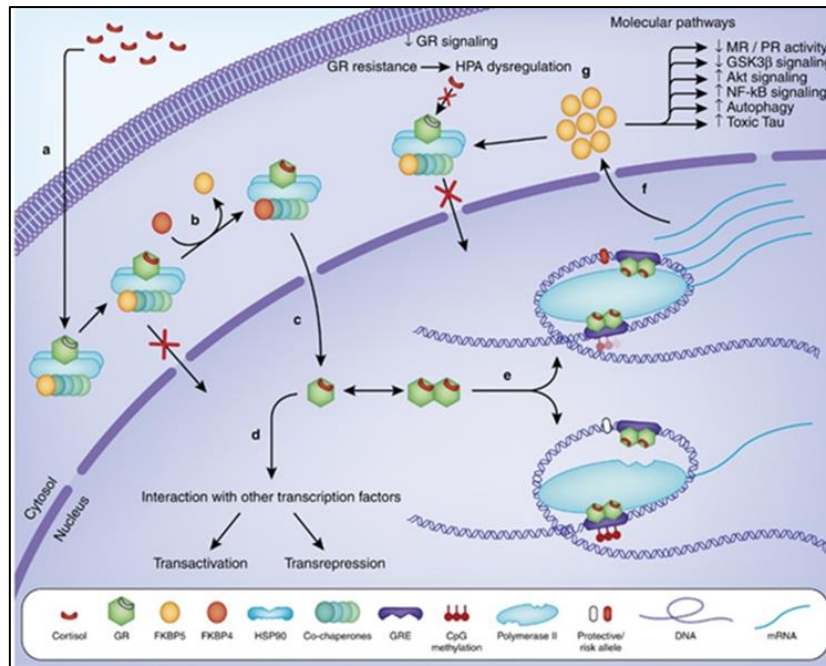


Figure 2-2 - Schematic diagram representing the molecular events involved in glucocorticoid mediated FKBP5 induction, the resulting intracellular negative feedback loop and the effects on other biological processes (Zannas et al., 2016)

Clinically, polymorphisms in FKBP5 have been reported to be associated with pre-traumatic dissociation in medically ill children, a symptom which is predictive of the development of PTSD (Zannas et al., 2016). A study in 700 highly traumatized inner city African-American individuals (Binder et al., 2008) demonstrated that as many as four FKBP5 gene polymorphisms may interact with childhood abuse as environmental factor to predict the ultimate severity of adult PTSD symptoms. These polymorphisms were also functional in individuals with both probable and actual PTSD and were associated with enhanced suppression of cortisol in response to dexamethasone (Binder et al., 2008). Considering the high rate of comorbidity between PTSD and depression, an interaction between the same four FKBP5 polymorphisms and childhood trauma was revealed to increase the risk for suicide among such individuals (Roy et al., 2010). Whereas it is often difficult to apply genetic variances in the diagnosis of psychiatric illness, it is noteworthy that differences in FKBP5 blood mRNA levels have been associated with PTSD in at least two separate studies (Koenen et al., 2005; Yehuda, 2009). Indeed, FKBP5 related stress dysregulation might be a risk factor for other stress-related psychiatric disorders as well, since the same alleles are over-represented in individuals with major depression, bipolar disorder and PTSD (Binder, 2009).

A number of such GxE interactions have been demonstrated and include interactions between catechol-O-methyl transferase (COMT), dopamine 2 (D₂) receptor, serotonin 2A (5HT_{2A}) receptor, and GABA_A receptor gene polymorphisms and exposure trauma (Binder, 2009; Nelson *et al.*, 2009). It can therefore be concluded that gene-environment interactions, as demonstrated between FKBP5 polymorphisms and childhood abuse, contribute to the pathogenesis of PTSD.

2.1.6 Epigenetics

Although demonstrating a causal link between environmental stressors and genetic susceptibility in the pathology of PTSD, studies of GxE interactions do not fully explain the importance of the timing of stress exposure during development. However, investigations into the epigenetic modification of DNA can however provide some insight (Skelton *et al.*, 2012). Epigenetic modification describes an environmentally induced change in DNA and its function, but not the structure of the gene as is true for polymorphisms (Skelton *et al.*, 2012). Such changes to genes are usually specific to critical developmental stages; they are stable, enduring and can be transmitted to subsequent generations (Meaney & Szyf, 2005). Briefly, the biological mechanism of epigenetic modification involves methylation of genetic cytosine, thereby preventing gene transcription and functional protein expression (Novik *et al.*, 2002). With respect to PTSD, evidence from animal studies demonstrates DNA methylation to be an important mechanism altering the activity of genes that regulate HPA-axis activity and that such methylation is often triggered by adverse early life events (Champagne & Meaney, 2001). Work by Meaney and colleagues (Meaney & Szyf, 2005) revealed a role for epigenetic mechanisms in mediating the effects of early environmental events on the behaviour of adult animals. They demonstrated that maternal care characterised by pronounced licking and grooming behaviour produced lower levels of cortisol in rat pups, but only if this occurs during a specific early developmental window (Meaney & Szyf, 2005). Furthermore, congruent with the clinical picture described above, the same pups demonstrated enhanced suppression of cortisol in response to dexamethasone and presented with a greater expression of the GR gene and hippocampal GRs (see section 2.3.4). These changes have been shown to result from hypomethylation in the promotor region of the hippocampal GR gene (Weaver *et al.*, 2002). Epigenetic mechanisms are also relevant for intrauterine development as evinced by lower salivary cortisol levels in infants of pregnant mothers diagnosed with PTSD as result of

exposure to the '9/11' attacks in New York. However, in congruence with previous findings (Meaney & Szyf, 2005), such alterations were only observed in infants of mothers exposed to trauma during the third trimester of gestation (Yehuda *et al.*, 2005). Further, vasopressin gene overexpression has been shown in a rodent model of early-life stress (ELS) (Murgatroyd *et al.*, 2009), while mice subjected to maternal separation have demonstrated consistent increases in corticosterone and vasopressin together with depressive behaviour that were subsequently reversed by a vasopressin antagonist (Skelton *et al.*, 2012). Since increased vasopressin signalling in brain areas involved in fear and anxiety processing results in elevated anxiety-like behaviour, this presents another epigenetic mechanism that may contribute to an increased vulnerability to develop PTSD.

In demonstrating that stable changes in the stress response result from early life interference and that these behavioural phenotypes are transmitted from one generation of female offspring to the next (Seckl and Meaney *et al.*, 2006), a clear molecular link between early environmental inference and gene expression and function is made (Skelton *et al.*, 2012). Importantly, the alterations observed in the findings of Meaney and Szyf (2005), i.e. increased GR expression, are congruent with those described in PTSD, and are incompatible with a unidimensional understanding of the role of GR sensitivity in the pathology of trauma-induced stress responses. As such, these findings offer proof of principle that environmental inferences are crucial to develop a robust model of PTSD that may contribute to our understanding of how pre-existing risk factors and their embroidered response to child- and adulthood trauma may manifest as an increased vulnerability to PTSD (Yehuda & Bierer, 2009).

2.2 The Neuroendocrinology of PTSD

Dysfunctional activity of multiple neurobiological pathways has been implicated in the pathology underlying PTSD symptomatology, 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.

2.2.1 The hypothalamic-pituitary-adrenal (HPA)-axis and the endocrine stress response

When exposed to stress the HPA-axis increases the secretion of corticotrophin releasing factor (CRF) from the hypothalamus that in turn stimulates the secretion of

adrenocorticotrophic hormone (ACTH) and subsequently cortisol from the adrenal cortices. Negative feedback control terminates the stress response and is critical in controlling the negative effects of excessive glucocorticoids on the brain and other organs. Stress and the associated increase in glucocorticoids provoke the release of glutamate where it plays an important role in learning and memory processes (Riedel *et al.*, 2003).

Low glutamate levels negatively influence memory formation while too high levels adversely affect memory due to its excitotoxic nature (Lowy *et al.*, 1995). Consequently, glutamate has a bimodal effect on memory function. Glutamate toxicity has been linked to hippocampal damage seen in PTSD patients (Stein-Behrens *et al.*, 1994) and is involved in stress and fear responding (Walker & Davis, 2000) and in various processes underlying anxiety (Baker & Azorlosa, 1996; Harvey & Shahid, 2012). Work in our laboratory has revealed the reciprocal involvement of glutamate and GABA in PTSD as well as the contributory role for inflammation (Harvey *et al.*, 2004b) thus illustrating their interactive role in the development of PTSD (Chambers *et al.*, 1999). A hyperglutamatergic state has been proposed for PTSD, while studies have confirmed that consolidation of traumatic memories is dependent on glutamatergic dependent signalling (Joca *et al.*, 2007; see also section 2.5.1).

Vasopressin and oxytocin are neuropeptides that also play an important role in the neurobiology of anxiety and stress (Hariri *et al.*, 2000). Altered vasopressin and oxytocin circadian fluctuations has been found in chronic anxiety disorders (McClung, 2013), while elevated plasma levels of vasopressin are evident in PTSD (De Kloet *et al.*, 2008). Elevated levels of vasopressin also correlate with avoidance symptoms in PTSD (De Kloet *et al.*, 2008) and has been found to enhance aggressiveness and anxiety in rats (Huber *et al.*, 2005). Vasopressin also plays a role in regulating ACTH secretion during psychological stress (Legros, 2001) and to mediate poor coping via the lowering of glucocorticoid levels (Legros, 2001). Oxytocin, on the other hand, facilitates glucocorticoid secretion (Windle *et al.*, 1997) and has anxiolytic effects in animals (Heinrichs *et al.*, 2003). This hormone is especially important in maintaining appropriate social behaviour and structure, especially in females via its role in the “tend and befriend” response (Taylor *et al.*, 2006). Therefore, elevated vasopressin and lowered oxytocin levels exert opposite effects on fear and anxiety related behaviour. Importantly the suppression of the cortisol response in PTSD is associated with the reduced stress coping due to high levels of vasopressin, altered cortisol release and low levels of

oxytocin, all leading to the development of anxiety and other stress related manifestations (Dedovic & Ngiam, 2015). These aspects will now be presented in more detail.

2.2.1.1 Hypothalamic-pituitary-adrenal (HPA) axis and the role of CRF, ACTH and corticosterone

The term stress was originally used to describe a physical or emotional stimulus that disrupts the balance of the internal environment, and that may cause pathology when exceeding a critical level (Cannon *et al.*, 1929). The purpose of the fear response is to eliminate the source of stress and to reinstate homeostasis so that adaptation takes place and survival is promoted (Carrasco & Van de Kar, 2003). For example, the stress response prepares for fight or flight, and enhances the memory of the potentially life-threatening event, thereby optimising the response to similar events in the future (McEwen, 2000; McEwen, 2007). Normally, reinstatement of the homeostatic balance follows successful adaptation. However, failure to cope with traumatic stress results in a new equilibrium, with significant neurobiological consequences. Chronic on-going stress, as seen in PTSD, causes maladaptive plasticity in the brain in which neurotransmitters, neuropeptides and hormones interact to produce structural and functional changes that are pathological (McEwen, 2000). For example, stress-induced damage to the hippocampus essentially underlies the poor negative feedback response on the HPA-axis to curb excessive release of stress hormones. The HPA-axis is the central coordinator of the neuroendocrine stress response systems and has been a major focus of scrutiny in patients with PTSD. In short, the HPA-axis is made up of endocrine hypothalamic components, including the anterior pituitary, as well as an effector organ, the adrenal glands. Upon exposure to stress, neurons in the hypothalamic paraventricular nucleus (PVN) secrete CRF from nerve terminals in the median eminence into the hypothalamus-hypophyseal portal circulation which stimulates the production and release of ACTH from the anterior pituitary (Figure 2-3). ACTH in turn stimulates the release of glucocorticoids from the adrenal cortex (Yehuda, 2006). Once activated, the HPA-axis triggers the production and release of glucocorticoids from the adrenal cortex and adrenaline from the adrenal medulla (Jacobson & Sapolsky, 1991; see Figure 2-4). The HPA system also triggers the release of the catecholamines dopamine and noradrenalin. Together these messengers play a central role not only in facilitating the stress response but also in laying down declarative memory and the contextual aspects of fear conditioning. Failure to curb the stress response is why PTSD can be regarded as a perpetual and inappropriate stress response that is damaging to the brain. As such, the HPA-axis negative feedback system is known to be altered in stress-related illnesses, such as PTSD and major depressive disorder (Heim & Nemeroff, 2001; Yehuda,

2002). Hypersensitive or enhanced negative feedback has been found in PTSD and has been described as a "sensitization of the inhibitory elements of the HPA-axis" (Liberzon *et al.*, 1997). The enhanced inhibitory effect that PTSD has on the HPA-axis causes patients with PTSD to have low cortisol levels despite having high CNS activity (Yehuda *et al.*, 1996; Baker *et al.*, 1999; Baker *et al.*, 2005). Animal models of PTSD, such as predator exposure (Cohen *et al.*, 2006b) and stress-restress (Harvey *et al.*, 2003b; Oosthuizen *et al.*, 2005a; Harvey *et al.*, 2006) have described a blunted HPA-axis response in these animals, resulting in an increased susceptibility to develop PTSD like symptoms. Therefore, sustained exposure to stressful events or reminders of traumatic experience may induce changes in HPA-axis responsivity that ultimately results in pronounced neuronal degeneration and cognitive impairment (Pynoos *et al.*, 1999), which can be seen as the abnormal arousal symptoms and memory alterations that occur in PTSD (Harvey *et al.*, 2006). Of importance within the context of this study is that a suppressed cortisol response is associated with reduced stress-coping, thereby linking altered circadian cortisol release to the development of an anxiety and/or stress-related disorder (Dedovic & Ngiam, 2015).

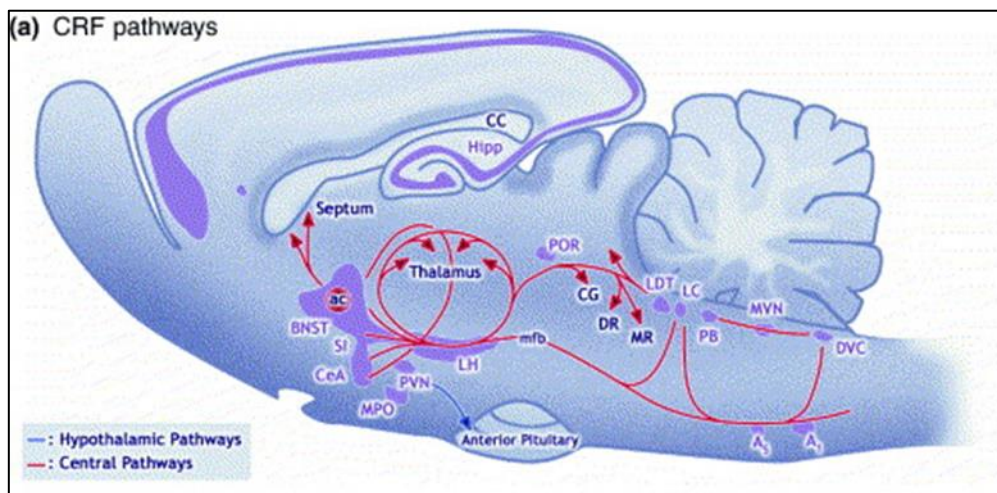


Figure 2-3 - Corticotrophin-releasing factor (CRF), vasopressin and neuropeptide Y (NPY) pathways in rat brain regions that process emotion and the response to stress (Henckens *et al.*, 2016)

2.2.1.2 The HPA-axis and the role of vasopressin and oxytocin

Vasopressin, or otherwise known as antidiuretic hormone (ADH), released from the posterior pituitary is known as a key regulator of the HPA-axis (see Figures 2-4 & 2-5), although on its own it has limited effects on ACTH secretion (Mizoguchi *et al.*, 2003). Functionally vasopressin potentiates the effects of CRF and ACTH release (Mizoguchi *et al.*, 2003). Repeated exposure to severe stressors provokes sustained HPA-axis activation and abrogates the glucocorticoid negative feedback loop (Mizoguchi *et al.*, 2003). This results in an increased density of vasopressin vesicles as well as the number of vasopressin and CRF co-expressing neurons in the paraventricular nucleus (Aguilera, 1998). While the regulating function of ADH on the HPA-axis during moderately stressful situations is beneficial, it is detrimental in states of chronic stress such as in PTSD (Mizoguchi *et al.*, 2003). A major component of the vasopressinergic system involved in glucocorticoid release originates from the AVP (arginine vasopressin, ADH)-containing cells of the paraventricular nucleus and project to the hypothalamic-hypophyseal portal vessels in the median eminence. Here they release AVP in the adenohypophysis where it acts synergistically with CRF as a secretagogue for ACTH (Aguilera & Rabadan-Diehl, 2000; Holmes *et al.*, 2003)

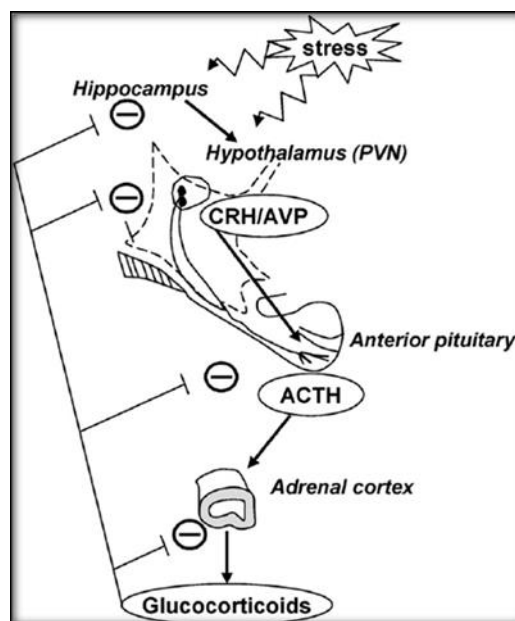


Figure 2-4 - Schematic diagram of the HPA-axis during stress, showing glucocorticoid-mediated feedback regulation of the HPA-axis. In reaction to stress and under the influence of hippocampal inputs, hypothalamic neurons synthesize and secrete corticotrophin-releasing hormone (CRH; CRF) and arginine-vasopressin (AVP). The latter neurohormones stimulate the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH) into the circulation. ACTH reaches the adrenal glands, where it stimulates cortisol / corticosterone secretion. The return to basal resting activity level is reached through negative feedback control triggered mainly by GR activation by cortisol / corticosterone in adrenal cortex, anterior pituitary and brain structures (Adapted from Lanfumey *et al.*, 2008)

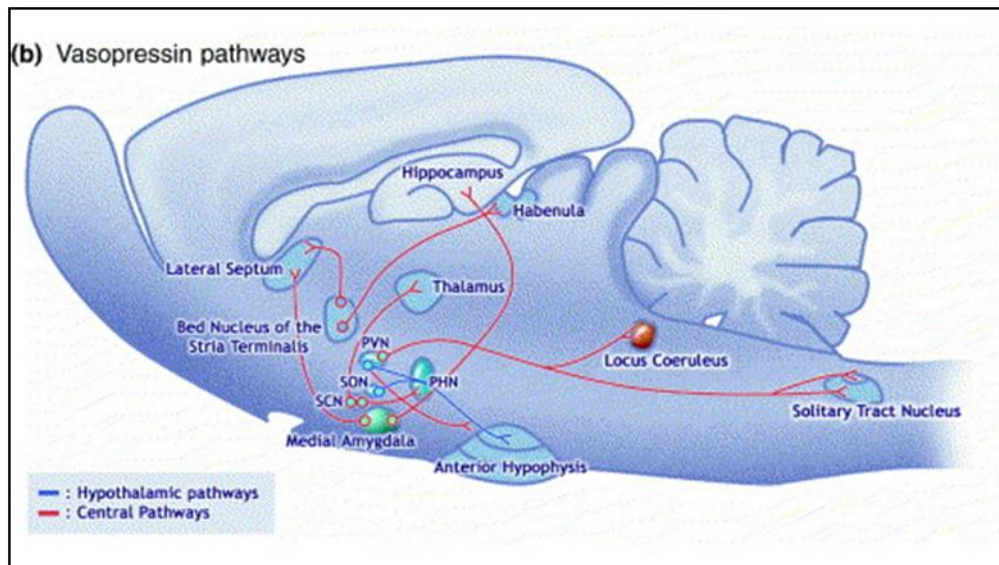


Figure 2-5 - Hypothalamic vasopressin pathways originate from the hypothalamus and project to the pituitary, or locus coeruleus (LC) and solitary tract nucleus (Henckens et al., 2016)

Given the functional relationship between ADH and the HPA-axis, attention has also been given to the possible role of oxytocin, which has been implicated in the pathophysiology of disturbed stress regulation as well as disrupted attachment and deficits in sociability, e.g. social phobia, mood disorders, borderline personality disorder, and PTSD (Taylor, 2006; see Figure 2-6). Oxytocin inhibits stress-induced activity of the HPA-axis (Neumann et al., 1999) by suppressing both sympathetic arousal and HPA-axis responses to stress. Higher oxytocin levels have also been associated with quicker HPA-axis recovery in women after an acute stress challenge. Oxytocin may also have a positive effect on social interaction and reduce the emotional numbing that is experienced by PTSD patients (Sailer et al., 2008). These effects can possibly be related to its stimulation of the nucleus accumbens, a brain region implicated in the experience of social reward (Sailer et al., 2008). In addition to the “fight or flight” response, humans also demonstrate a “tend and befriend” response to stress that seems to be dependent on oxytocin (Taylor, 2006). Tending pertains to nurturing activities intended to promote safety and reduce distress; befriending is forming and maintaining social networks that may assist in stressful situations (Taylor, 2006). As PTSD is characterised by deficits in social functioning and stress regulation and considering the role of oxytocin in the regulation of both stress and social functioning, the peptide is realising significant interest as a possible therapeutic intervention for PTSD (Taylor, 2006).

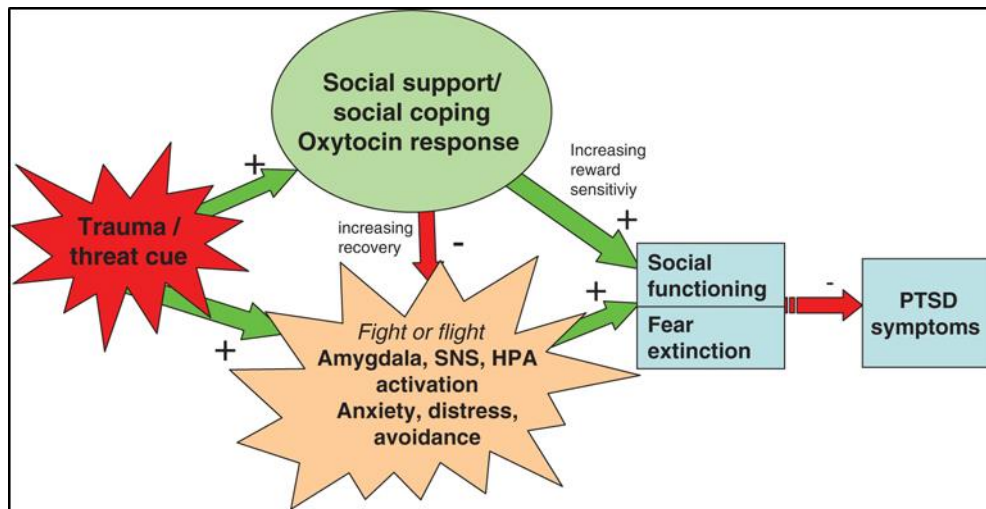


Figure 2-6 - Oxytocin and the HPA-axis

Oxytocin inhibits stress-induced activity of the HPA-axis (Neumann et al., 1999) by suppressing both sympathetic arousal and HPA-axis responses to stress and therefore the oxytocin system is important for increasing fear extinction and social functioning after trauma. Adapted from (Olf, 2012)

2.3 The Neurochemistry of PTSD

The following will be discussed in this review the noradrenergic, serotonergic, dopaminergic, GABA and glutamate systems role in PTSD.

2.3.1 The Noradrenergic System

Noradrenalin (NA) is one of the principle mediators of the central nervous system (CNS) and autonomic stress response. The majority of forebrain NA is provided by the locus coeruleus, a small pontine nucleus (Charney, 2004). This structure innervates the amygdala, hippocampus, and the infra limbic medial prefrontal cortex (IL-mPFC; Mueller et al., 2008) which are involved in extinction learning and retrieval (Charney, 2004). Activation of the noradrenergic system enhances memory function since noradrenalin increase neural firing in the hippocampus, affirming its prominent role in the enhancement of memory storage (Singewald et al., 2015). Noradrenergic brain systems are involved in the neural mechanisms of fear conditioning, extinction as well as sensitization (Holmes & Quirk, 2010). Fear conditioning is modulated by the release of noradrenalin in brain regions involved in fear conditioning. Noradrenalin enhances neuronal excitability in extinction related brain regions such as the infralimbic prefrontal cortex (IL) (Mueller et al., 2008) and successful fear extinction is associated with enhanced extracellular levels of noradrenalin in the mPFC (Hugues et al., 2007). Extensive research indicates that the stress-related neurotransmitter noradrenalin (NA) strengthens the formation of aversive memories (McGaugh, 2004).

Enhancing noradrenalin levels with administration of noradrenalin (Singewald *et al.*, 2015) or with compounds such as yohimbine (Nirogi *et al.*, 2012) has shown to enhance fear extinction. Thus, arousal-evoked NA release strengthens acquisition of aversive memories via β -receptor signalling (Singewald *et al.*, 2015). On the other hand, depleting central noradrenalin or lesioning ascending noradrenalin projections from the locus coeruleus, impairs extinction, with systemic α -1 or β adrenoceptor blockade showing the same effects on extinction. Blockade of NA signalling through noradrenergic β -receptors results in a loss of this enhancement in both animals (Ji *et al.*, 2003) and humans (Grillon *et al.*, 2004). There has been interest in the clinical utility of targeting noradrenergic mechanisms to augment extinction. Here, we focus on α_2 -adrenoceptors given the recent clinical findings supporting the utility of α_2 -adrenoceptor antagonists in improving extinction. In rodents, blocking α_2 -adrenoceptors (e.g. with yohimbine), that function as auto-receptors on locus coeruleus neurons, increases locus coeruleus activity and noradrenalin release in terminal regions (Singewald & Philippu, 1998; Singewald *et al.*, 2015).

NA acts at multiple receptors in target tissue where its effects are mediated via postsynaptic α_1 , β_1 , and β_2 receptors and α_2 receptors, the latter serving as a presynaptic auto receptor inhibiting NA release (Carrasco & Van de Kar, 2003). These receptor subtypes are distributed across various brain regions (Berridge & Waterhouse, 2003) where NA serves as a warning system and assists in the appropriate response to relevant stimuli while suppressing that to irrelevant stimuli (Vermetten & Bremner, 2002). NA therefore acts as an alarm reaction that allows for optimal coping in the face of adversity. The LC system has multiple roles in regulating arousal (Haden *et al.*, 2007) and autonomic stress responses as well as promoting the encoding of emotional memories, the latter-mentioned impacting on alertness, vigilance, selective attention and cardiovascular responses (Southwick *et al.*, 1999). Therefore, NA is a suitable candidate in studying the pathophysiology of PTSD.

The noradrenergic pathway is involved in memory processes, in particular fear conditioning. Fear conditioning tasks causes the release of noradrenalin by activation of vagal afferents to the nucleus tractus solitarius (NTS). Noradrenergic neurons in the NTS project directly to the basolateral amygdala (BLA) as well as indirectly, *via* the nucleus paragigantocellularis (PGi) to the LC. The latter in turn projects to the BLA. Noradrenalin released from the NTS and the LC activates postsynaptic β and α_1 -adrenoceptors with activation of these receptors in the BLA leads to the activation of the adenosine 3',5'-cyclic monophosphate (cAMP) pathway

resulting in cAMP-dependent protein kinase (PKA) formation (McGaugh, 2002; Roozendaal *et al.*, 2002). The latter activates long-term potentiation (LTP) in the amygdala (Huang & Kandel, 1998), an important factor in fear conditioning (Josselyn *et al.*, 2001; see Figure 2-7).

In PTSD, emotional events are associated with high levels of NA release in brain areas involved in learning and memory (McGaugh *et al.*, 2002; McGaugh, 2004). Emotional memories are mainly influenced by noradrenergic $\alpha_{1/2}$ and β_2 receptors (Cahill *et al.*, 1996). In the basolateral amygdala, emotionally aroused noradrenergic activation tends to strengthen memory consolidation in the hippocampus which is responsible for arranging contextual fear memory (McGaugh & Roozendaal, 2002). Therefore, suitable curbing of the noradrenergic system could be an important neurobiological target in treating PTSD. Various clinical and preclinical (Bryant *et al.*, 2009; Holbrook *et al.*, 2010) studies have discovered the potential of targeting noradrenergic receptors, especially α_1 (e.g. with prazosin) and β_1 - (e.g. with propranolol) antagonists to treat PTSD (Uys *et al.*, 2017). At least three studies have shown evidence of the role of noradrenaline in PTSD. Increased urinary levels of noradrenalin 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 (Lemieux & Coe, 1995). Higher levels of NA have also been observed in the cerebrospinal fluid of male combat veterans with chronic PTSD while also correlating with symptom severity (Geraciotti Jr *et al.*, 2001). Studies showing hypersensitivity of adrenergic receptors in patients with chronic PTSD (Southwick *et al.*, 1997b) also provides evidence for a hyperadrenergic state in PTSD. However other studies showed a reduction in platelet α_2 -adrenergic receptors in PTSD patients, which will further amplify the response to already elevated NA levels (Gurguis *et al.*, 1999).

Noradrenergic activation in the BLA stimulates glutamatergic mechanisms in the BLA to facilitate NMDA-dependent plasticity (Lennartz *et al.*, 1996). Studies by Roesler and colleagues (1999) showed that an NMDA-antagonist induces memory deficits in rats which cannot be reversed by noradrenalin, suggesting that the modulatory effects of noradrenalin on memory consolidation may involve NMDA-dependent mechanisms. However, NA may also modulate glucocorticoid actions and in this way affect stress responding, and *vice versa*.

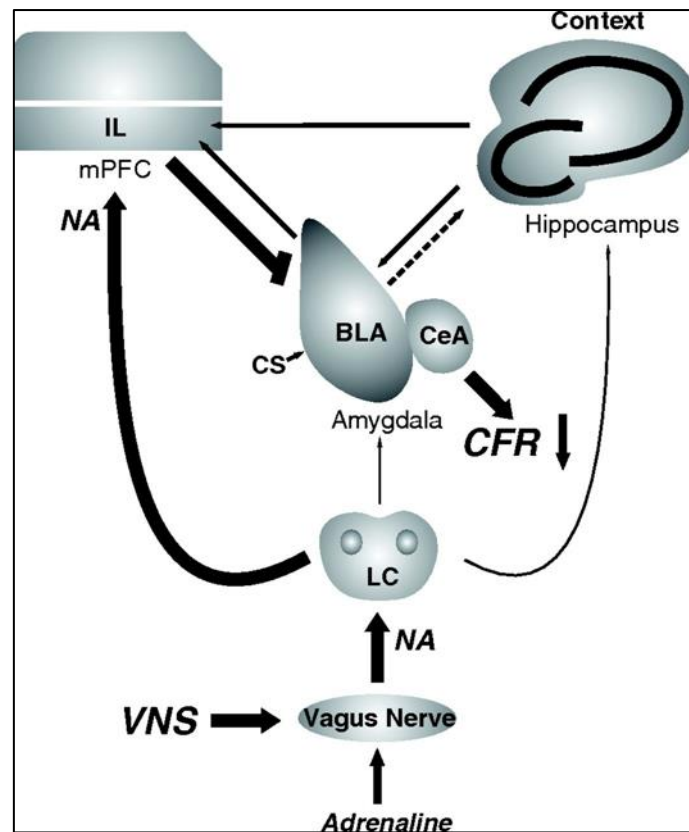


Figure 2-7 - Vagal nerve and adrenaline interactions during stress

Vagal nerve stimulation (VNS) during fear extinction leads to noradrenalin (NA) release in the amygdala, hippocampus, and medial prefrontal cortex (mPFC). Adrenaline, which is released during an emotionally charged experience, communicates with the central nervous system (CNS) via activation of the adrenergic β -receptors on the vagal nerve resulting in activation of locus coeruleus (LC) neurons with NA. The LC-evoked NA release during extinction learning, where a conditioned stimulus (CS) is presented without the unconditioned stimulus, results in a sustained adrenergic activation of the infra limbic region (IL) of the mPFC (thick arrows). Thereby, plasticity in this area is enhanced (Mueller et al., 2008) leading to increased inhibition of the basolateral amygdala (BLA). Upon retrieval, decreased activity in the amygdala and its output structure, the central nucleus (CeA), results in a reduced conditioned fear response (CFR). The mPFC integrates context-dependent information from the hippocampus and amygdala (thin arrows) to execute appropriate behaviour (Quirk & Mueller, 2008; Mueller & Cahill, 2010).

Glucocorticoid hormones freely enter the brain and bind to two intracellular types of adrenal steroid receptors (Reul & Kloet, 1985). Extensive evidence indicates that the low-affinity glucocorticoid receptors (GRs), and not the high-affinity mineralocorticoid receptors, are involved in mediating glucocorticoid effects on memory consolidation (Reul & Kloet, 1985). Noradrenergic cell groups in the NTS and the LC express high densities of glucocorticoid receptors (Härfstrand et al., 1986). Glucocorticoids freely enter the brain and bind to GRs in noradrenergic cell bodies in the NTS to potentiate noradrenalin release in the BLA, as well as postsynaptically in BLA neurons to facilitate the noradrenalin signal cascade (Härfstrand et al., 1986; see Figure 2-8). These stress hormone effects on noradrenergic activation in the BLA are required for regulating memory consolidation in other brain regions. Extensive evidence indicates that epinephrine affects memory consolidation by involving noradrenergic activation in the amygdala (de Kloet et al., 1999). Post-training activation of GRs on

noradrenergic cell groups in the NTS induces dose-dependent memory enhancement (de Kloet *et al.*, 1999; Roozendaal, 2000). It has been 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). Other studies showed a dose-dependent enhancement of memory when glucocorticoid receptors on noradrenergic cell groups in rat NTS were activated after a training task (de Kloet *et al.*, 1999; Roozendaal, 2000). This enhancement was blocked by subsequent infusion of a β -receptor antagonist into the basolateral amygdala (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 (McGaugh & Roozendaal, 2002).

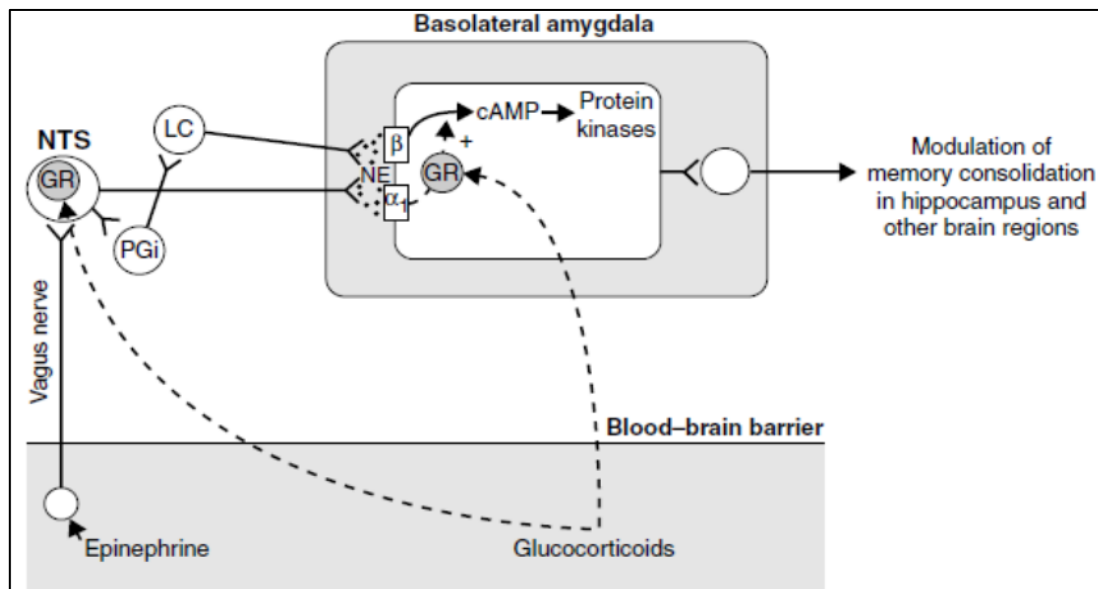


Figure 2-8 - Interactions of adrenal stress hormones with the noradrenergic system in the BLA in modulating memory consolidation (McGaugh & Roozendaal, 2002); BLA: basal lateral amygdala

2.3.1.1 A new way forward – antagonizing the α_{2C} adrenoceptor

Alpha 2C adrenoceptors (α_{2C} AR) regulate a diverse range of physiological processes, including vigilance, anxiety, pain and cardiovascular function (Ruffolo Jr *et al.*, 1988; Starke, 2001). Work on non-selective alpha-2 antagonists have shown that although both α_{2A} and α_{2C} auto receptors inhibit the release of NA (Hein *et al.* 1999), this effect is less prominent for α_{2C} compared to α_{2A} receptors (Bücheler *et al.* 2002). However, NA exhibits a higher affinity for the α_{2C} receptor and inhibits its own release at lower endogenous NA concentrations compared to α_{2A} ARs (Bücheler *et al.* 2002). These qualities are of importance when

considering the use of selective α_{2C} receptor antagonists in animal models characterised by hyperadrenergic states, e.g. PTSD and schizophrenia (Yamamoto, Hornykiewicz 2004). Interestingly, and in contrast to the α_{2A} receptor, α_{2C} receptors do not undergo agonist-induced phosphorylation (Jewell-Motz, Liggett 1996) and do not appear to down-regulate in response to chronic agonist treatment (Eason, Liggett 1992).

α_{2C} ARs are expressed in the striatum, hippocampus, and olfactory tube (Scheinin *et al.*, 1994; Winzer-Serhan & Leslie, 1997), all of which are important role players in cognitive functioning and mood regulation (Sapolsky, 2001). It is therefore possible that the α_{2C} AR specifically may play a role in the neurobiology of neuropsychiatric disorders. Functional differences between α_{2A} and α_{2C} receptors have been demonstrated in transgenic mouse models with distinct and opposite roles in spatial navigation and behavioural despair having been elucidated, whereby α_{2C} receptor antagonism promotes spatial navigation ability and memory (Björklund *et al.* 1999, Björklund *et al.* 2000). Therefore, selective α_{2C} antagonism (but not α_{2A}) may elicit bolstered antidepressant-like effects compared to nonselective α_2 modulating drugs. In fact, the selective α_{2C} AR-antagonists, ORM-10921 and JP-1302, have demonstrated significant antidepressant-like activity in Sprague Dawley and Han-Wistar rats, respectively (Sallinen *et al.*, 2007; Sallinen *et al.*, 2013). It has further been demonstrated that ORM-10921, in addition to its antidepressant-like effects, also has antipsychotic and pro-cognitive effects in translational models of depression and schizophrenia (Uys *et al.*, 2017). However, its putative anxiolytic effects, especially in a translational model of PTSD, have not yet been investigated. Indeed, as alluded to above, over-expression of α_{2C} receptors (α_{2C} OE), which mimics the effect of chronic subtype selective agonist treatment (Scheinin *et al.*, 2001), increases immobility (depressive-like behaviour) as well as corticosterone levels following repeated stress. On the other hand, α_{2C} receptor knockout (α_{2C} KO) mice, which mimic the effects of chronic administration of a subtype selective antagonist, are protected against these effects (Sallinen *et al.*, 2007). Interestingly, α_{2C} KO mice also present with decreased 5HT, 5-hydroxyindoleacetic acid (5HIAA), 3-4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) concentrations (Sallinen *et al.*, 1999). The α_{2C} AR has also been associated in α_2 -autoreceptor-mediated modulation of cortical and hippocampal dopamine (DA) and NA synthesis via feedback inhibition on tyrosine hydroxylase, which converts tyrosine to the DA precursor 3,4-dihydroxyphenylalanine (DOPA) (Uys *et al.*, 2017).

Studies done by Esteban *et al.* (1996) used early subtype-specific agonists and antagonists to measure levels of NA and DOPA in rodent hippocampus and cerebral cortex, with $\alpha_{2B/C}$ AR antagonists increasing synthesis of DOPA and $\alpha_{2B/C}$ AR agonists decreasing its synthesis (Uys *et al.*, 2017). Even though the ligands used were $\alpha_{2B/C}$ AR specific ligands, the expression of α_{2B} ARs is limited to the hypothalamus and does not seem to contribute to auto- and heteroreceptor function in the CNS (Trendelenburg *et al.*, 2001). Esteban *et al.* (1996) also reported that α_{2A} AR specific antagonism and agonism were without any effects on DOPA. α_{2C} AR selective antagonism could, however, play a role in increasing DA and NA levels and thus be of benefit in the treatment of neuropsychiatric illness.

The heightened expression of α_{2C} ARs in the striatum allows the striatum to modulate presynaptic DA release and DA-mediated behaviours (Bücheler *et al.*, 2002; Uys *et al.*, 2017). Studies done by Zhang and co-workers provided early evidence for the ability of DA to function as an activating ligand on striatal α_{2C} ARs (Zhang *et al.*, 1999), while other studies used (Sallinen *et al.*, 2013) ORM-10921 to show increased in vitro α_{2C} AR potency and selectivity ratios in the presence of DA (Figure 2-9). Indeed, it was shown that ORM-10921 increases extracellular DA levels in the rodent prefrontal cortex (PFC) (Sallinen *et al.*, 2013). Early studies indicated changes in brain DA metabolism in α_{2C} -KO and α_{2C} -OE mice (Sallinen *et al.*, 1997) which supports the correlation between DA activity and α_{2C} AR activity. In this study the α_{2C} -OE mice show higher levels of the DA metabolite homovanillic acid (HVA) in the frontal cortex but not in the striatum compared to wild-type controls, whereas α_{2C} -KO animals showed lower HVA concentrations in the striatum (Sallinen *et al.*, 1997), although not in the frontal cortex. The above findings suggest decreased striatal DA turnover in response to α_{2C} AR deactivation and increased cortical DA turnover in response to α_{2C} AR stimulation indicating an important relationship that exists between DA and the α_{2C} AR. The therapeutic potential of this can be realized in the targeting of α_{2C} ARs in disorders characterized by mesolimbic-cortical DA imbalance and it can be hypothesized that α_{2C} receptor antagonists may be of therapeutic benefit in the treatment of stress related disorders such as PTSD, a theory that will be addressed in the current investigation.

2.3.2 The dopaminergic system

There are four major dopaminergic pathways in the brain, namely the mesolimbic-, mesocortical-, nigrostriatal-, and tubero-infundibular pathways. Preclinical studies have demonstrated that stress has a negative impact on the normal physiology of the central dopaminergic system (Pani *et al.*, 2000) that could lead to aggravated fear conditioning (Debiec & LeDoux, 2006). Indeed, dopaminergic innervation of the medial PFC seems to be more susceptible to mild stress compared to other dopaminergic pathways that are only affected by severe stressors. As the mesolimbic and mesocortical dopaminergic systems are important for normal cognitive processing, a decrease in dopaminergic activity in the PFC has been suggested to contribute to hypervigilance and susceptibility to trauma related stimuli (Harvey *et al.*, 2006). Furthermore, dopamine also influences memory processing and may interfere with normal extinction or coping mechanisms in patients with PTSD (Stam, 2007). The actions of DA are mediated by D₁ or D₂ receptors (Jaber *et al.*, 1996). In the PFC, abundant expression of D₁ receptors may play a pathological role in PTSD, since high hyperdopaminergic activity on D₁-receptors transpires as impaired PFC function (Arnsten, 2009).

Reward pathways in the brain were first described by Olds and Milner (1954). They observed that when electrodes were placed in certain areas of the brain, rats would actively self-stimulate these areas, often to the exclusion of all other activities, including eating. The circuits involved in this process have been referred to as the reward system. The dopaminergic reward pathways progress from the ventral tegmental area (VTA) to the nucleus accumbens, olfactory tubercle, ventral striatum and frontal cortex (Wise & Rompre, 1989). The dopaminergic system is important for hedonic impact and reward learning and a decrease in dopaminergic activity in the prefrontal cortex has been suggested to affect the ability to develop coping strategies in dealing with a trauma and so contributing to hypervigilance and susceptibility to trauma related stimuli (Harvey *et al.*, 2006).

The specific role for DA in a model of PTSD has not been fully clarified, although Harvey and colleagues showed that DA responds differently immediately post-stressor and 7 days after a stressor in a PTSD model. (Harvey *et al.*, 2006). However, it has been reported that high urinary excretion of DA and its metabolite (homovanillic acid; HVA) occurs in patients with PTSD (Glover *et al.*, 2003). At the CNS level, mesolimbic DA plays a vital role in processing of rewards while DA has been shown to be involved in fear conditioning (Glover *et al.*, 2003). Evidence suggests that humans exposed to trauma demonstrate increased release of

mesolimbic DA which in turn has an impact on the HPA-axis response (Heim & Nemeroff, 2009). Thus, dysfunction of the central dopaminergic activity may be the cause of cognitive impairment, reduced extinction of conditioned fear, coping deficits and abnormal motivational and rewards and so contributing to re-experiencing, hyperarousal and avoidance symptoms as seen in PTSD patients due to relationships that exist between DA the α_{2C} AR as discussed above (section 2.3.1.1; Figure 2-9; Charney, 2004). In addition, sulpiride, a selective D_2 -receptor antagonist, facilitates fear memory extinction while quinpirole, a full D_2 -receptor agonist, blocks the acquisition of second-order fear conditioning (Nader & LeDoux, 1999). Suggesting that that dopamine D_2 -receptors are necessary for fear learning (Fadok *et al.*, 2009), while DA is necessary for cue-dependent fear conditioning. As a result, D_2 -receptor stimulation would strengthen fear conditioning, whereas a D_2 -antagonist should inhibit fear conditioning.

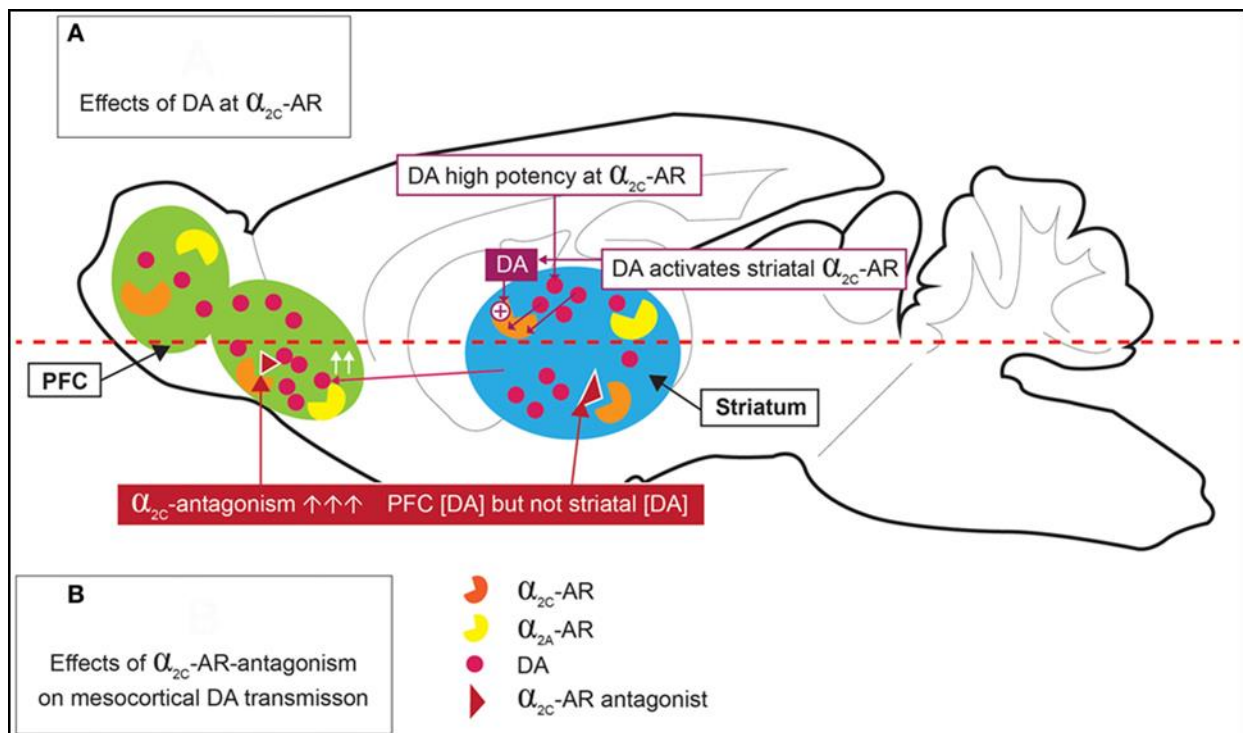


Figure 2-9 - Dopamine (DA) stimulation of α_{2C} ARs, and effects of α_{2C} AR-antagonism on mesocortical DA. See sections 2.3.1 and 2.3.2 for more detail. (Uys *et al.*, 2017)

2.3.3 The serotonergic system

As is true for the noradrenergic system, serotonergic neurons originating in the dorsal and medial raphe nuclei in the brainstem also project to the amygdala, hippocampus, hypothalamic nuclei and PFC (Lechin *et al.*, 2006). In this regard, the serotonergic system has been implicated in the pathophysiology of anxiety, arousal, vigilance, aggression, mood as well as in the modulation of affective and stress responses and neuroendocrine function (Raymond *et al.*, 2001). Further, the serotonergic system interacts with CRF and NA to coordinate the stress response and may, depending on the nature of the stressor, contribute to either facilitation or inhibition of both basal and stress-induced glucocorticoid secretion (Lowry, 2002). Indeed, stimulation of 5HT_{2A} receptors in the amygdala and hippocampus or decreased stimulation of 5HT_{1A} receptors in the hippocampus may result in anxiety (Vermetten & Bremner, 2002). Dysregulation of serotonergic neurotransmission may also contribute to the abnormal neuroendocrine stress response associated with PTSD (Vermetten & Bremner, 2002) (see Figure 2-10). These effects may possibly be explained by a decrease in serotonergic neurotransmission in PTSD as serotonin acts as a counter-regulatory neurotransmitter that reduces noradrenergic firing in the LC (Newport & Nemeroff, 2000). That SSRIs are the drugs of choice for PTSD provides convincing support for the importance of the serotonergic system in the stress response and PTSD. However, the extent of such involvement in PTSD and its interaction with other systems remains to be elucidated. SNRIs e.g. venlafaxine, dexvenlafaxine, duloxetine, and milnacipran, bind to norepinephrine and 5HT transporters and inhibit the reuptake of NA and 5HT from the synaptic cleft (Zhou, 2004). SNRIs have the advantage of acting on different areas of monoamine functioning simultaneously and may therefore address different neurobiological constructs. For example, aggression is ascribed to 5HTergic mechanisms (Healy & McMonagle, 1997), while deficits in motivation may more be associated with altered noradrenergic signalling (Jaffee *et al.*, 2007).

Environmental information about threatening stimuli is relayed to the BLA, a structure with glutamatergic projections to a wide range of limbic structures involved in the mediation of overt fear and anxiety behaviours (Walker *et al.*, 2003). Importantly, BLA output is modulated by 5HT (Bagdy *et al.*, 2001), with 5HT_{2C} receptor agonists anxiogenic (Bagdy *et al.*, 2001) leading to activation in BLA projection regions (Campbell & Merchant, 2003), and 5HT_{2C} receptor antagonists acting as anxiolytics (Bagdy *et al.*, 2001). A study by Strong *et al.* (2009) demonstrated that a systemic 5HT_{2C} antagonist blocked while 5HT_{2C} agonists mimicked the effects of inescapable tail shock on freezing and escape behaviour.

This suggests a role for 5HT in PTSD, but since the SSRIs also have a modulatory effect on the locus coeruleus/noradrenergic system (Hageman *et al.*, 2001), the beneficial action of these drugs in PTSD may be indirect or multi-targeted (Szabo *et al.*, 1999). Other studies suggest that PTSD is accompanied by changes in the 5HT transporters (Bagdy *et al.*, 2001). In this study Vietnam veterans with PTSD were administered the mixed serotonin agonist meta-chlorophenylpiperazine (mCPP), followed by behavioural and cardiovascular assessments. As such, one-third of patients with PTSD receiving mCPP experienced panic attacks and presented with significantly more anxiety-related and PTSD symptoms (e.g. flashbacks, hypervigilance, intrusive thoughts) compared to healthy controls (Southwick *et al.*, 1997a). Indeed, 5HT₂ receptors mediate the anxiogenic effects of serotonin, such as fear and anxiety (Zhang & Stackman Jr, 2015), while 5HT₁ receptors mediate its anxiolytic effects, facilitate extinction, and suppress encoding of spatial memory (Koenig *et al.*, 2008).

2.3.4 The GABA and glutamatergic system

GABA and glutamate are involved in the progression of actual memory registration and in encoding fear and emotional memory (Corcoran & Maren, 2001; DeLorey *et al.*, 2001; see section 2.5). Primary sensory transmission involves two components: the excitatory amino acid glutamate and the inhibitory amino acid γ -amino butyric acid (GABA). Glutamate is the primary excitatory transmitter in the brain and plays an important role in the process of consciousness and memory (Collingridge & Bliss, 1995). GABA is the main inhibitory neurotransmitter in the central nervous system and exerts its effects through binding to the GABA_A receptor, the most common receptor in the brain, which in turn inhibits the activation of NA, 5HT, DA, glutamate and other transmitters systems through GABA inter-neuronal connections (Kalueff & Nutt, 2007; see Figure 2-10).

GABAergic transmission is also thought to be important in the precise regulation of fear conditioning (Makkar *et al.*, 2010) and has been implicated in the pathogenesis of anxiety, depression, and insomnia, which are among the disturbances seen in PTSD (Harvey *et al.*, 2004b). Further, GABA regulates the duration and intensity of the central hyperadrenergic response in times of extreme stress or trauma (Corcoran & Maren, 2001). Rea and colleagues found reduced GABAergic signalling 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 & Lattal, 2011;

Sierra-Mercado *et al.*, 2011). Pronounced GABAergic activity would therefore lead to diminished consolidation, expression and extinction of conditioned fear (Mahan & Ressler, 2012).

Glutamate is the key excitatory neurotransmitter in the brain and is crucial in the extinction and consolidation of conditioned fear (Bast *et al.*, 2001; Harvey *et al.*, 2004b). Exposure to stressors and the release or administration of glucocorticoids increases the release of glutamate in the brain (Bast *et al.*, 2001). Glutamatergic transmission in the BLA occurs after fear conditioning in rats (Lin *et al.*, 2010) with glutamatergic neurons projecting from the cortical structures and sensory thalamic structures, the hippocampus and PFC to the BLA (Bast *et al.*, 2001; Pape & Pare, 2010). 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, 2012). The metabotropic receptors modulate synaptic plasticity via G-protein coupled signal transduction (Rumpel *et al.*, 2005), whilst the ionotropic glutamate receptors are important in the mediation of synaptic plasticity for the formation of long-term fear memories (Rumpel *et al.*, 2005). Glutamate binds to several of the excitatory amino acid receptors, one of which is the N-methyl-aspartate (NMDA) receptor; recent evidence proposes that fear conditioning activates NMDA receptors, causing a cascade of signal transduction mechanisms which result in the synaptic upregulation of AMPA receptors, in turn resulting in long term potentiation LTP and heightened responsiveness to a conditioned stimulus (Rumpel *et al.*, 2005; Nedelescu *et al.*, 2010). LTP plausibly contributes to consolidation of trauma memories in PTSD. Translational animal models of PTSD have demonstrated robust involvement of various aspects of glutamate signalling (Harvey & Shahid, 2012), including that of the nitric oxide pathway (Harvey *et al.*, 2004b; Harvey *et al.*, 2005b; Oosthuizen *et al.*, 2005b; Harvey & Shahid, 2012). The partial NMDA-receptor antagonist, D-cycloserine, has been shown to improve the extinction of fear in rodents and in phobic individuals undergoing exposure therapy (Heim & Nemeroff, 2009). However, its efficacy in enhancing the outcome of exposure therapy in PTSD remains to be studied. In addition to glutamate's role in memory and learning, overexposure of glutamate is associated with excitotoxicity and could contribute to the loss of neurons in the PFC and hippocampus, as described in PTSD patients (Heim & Nemeroff, 2009). Elevated glucocorticoids increase the expression or sensitivity of NMDA receptors which may sensitize the brain to excitotoxic insults (Heim & Nemeroff, 2009).

The time-dependent stress (TDS) model, a useful animal model of PTSD, has demonstrated attenuated GABA levels together with altered glutamatergic NMDA receptor binding in the rat hippocampus (Harvey *et al.*, 2004b). Thus, the combined change in glutamatergic and GABAergic signalling 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.*, 2005b).

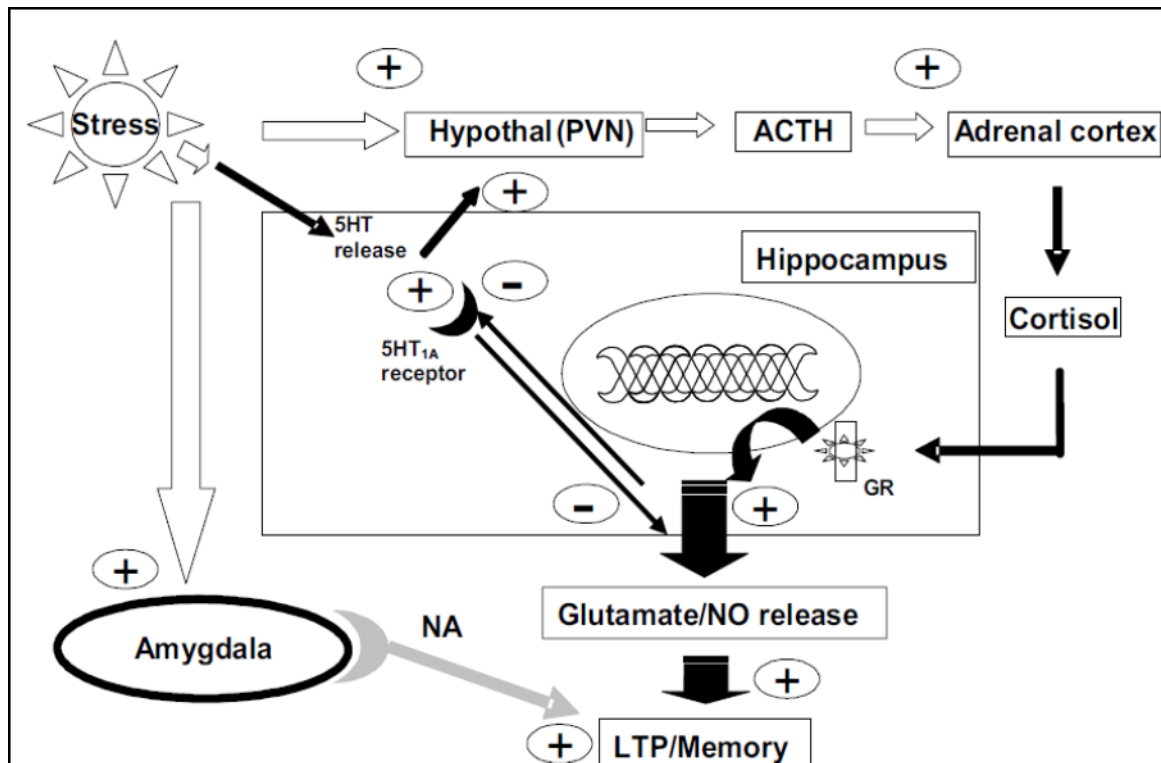


Figure 2-10 – Interplay between glutamatergic and serotonergic signalling in PTSD

Acute moderate to high stress activates serotonergic neurons in the hippocampus to release 5HT. This then activates postsynaptic 5HT_{1A} receptors that inhibit the process of hippocampal LTP, dependent on glutamate-NO mechanisms, and effectively suppresses the encoding of excessive emotional memory. On the other hand, 5HT will also stimulate the release of ACTH from the hypothalamus, resulting in the secretion of glucocorticoids. Activation of the glucocorticoid receptor in the hippocampus inhibits the expression of 5HT_{1A} receptors, preventing 5HT_{1A} receptors from inhibiting LTP-mediated hippocampal memory formation, while the subsequent release of glutamate evoked by glucocorticoids will further promote inappropriate memory consolidation. As the stressor increases in intensity, the amygdala is activated, which further activates hypothalamic glucocorticoid release and strengthens hippocampal LTP via noradrenergic mechanisms, while the absence of adequate 5HT_{1A}-mediated suppression of hippocampal LTP, together with excessive glutamatergic activity, all combine to promote the consolidation of emotional-driven memories (Oosthuizen *et al.*, 2005b).

2.4 The Neuroanatomy of PTSD

2.4.1 The frontal cortex

The prefrontal cortex (PFC) is associated with executive functioning and facilitates reasoning, planning, emotional processing as well as movement and some forms of speech (Clark *et al.*, 2005; see Figure 2-11). The PFC also processes feelings of frustration, tension, and anxiety while interpreting ongoing events and predicting outcomes of future similar situations. The prefrontal cortex has broad connections with other cortical and subcortical regions. While the ventromedial PFC shares broad connections with subcortical structures such as the amygdala, hypothalamus and the nucleus accumbens that regulate emotional responses (Goldman-Rakic, 1987), the dorsolateral PFC connects to the sensory and motor cortices that in turn regulates thought, action and attention. As a result of this functional organization, the PFC coordinates behavioural regulation and emotion under normal non-stressful circumstances. In PTSD however, this process is significantly attenuated during and following exposure to reminders of traumatic events or fearful stimuli (Bremner, 1999; Shin *et al.*, 2004; Shin *et al.*, 2005). Moreover, by virtue of its mediation of response implementation, memory retrieval, and emotional evaluation, the PFC is believed to contribute to the interchange between emotions and memory consolidation (Miller & Cohen, 2001). Congruent with these hypotheses, functional imaging studies have shown decreased activation of the medial PFC in PTSD patients responding to traumatic stimuli (Bremner, 1999; Shin *et al.*, 2004). A meta-analysis of structural brain abnormalities in PTSD also revealed the condition to be associated with significant reductions in prefrontal lobe volumes (Shin *et al.*, 2004; Karl *et al.*, 2006). Pre-clinical investigations also revealed decreased activation of the anterior cingulate cortex (ACC) and the subcallosal gyrus (SG) in patients with PTSD; these brain regions play an important role in curbing the manifestations of arousal, viz. aggression, reckless or self-destructive behaviour, sleep disturbances, and hypervigilance.

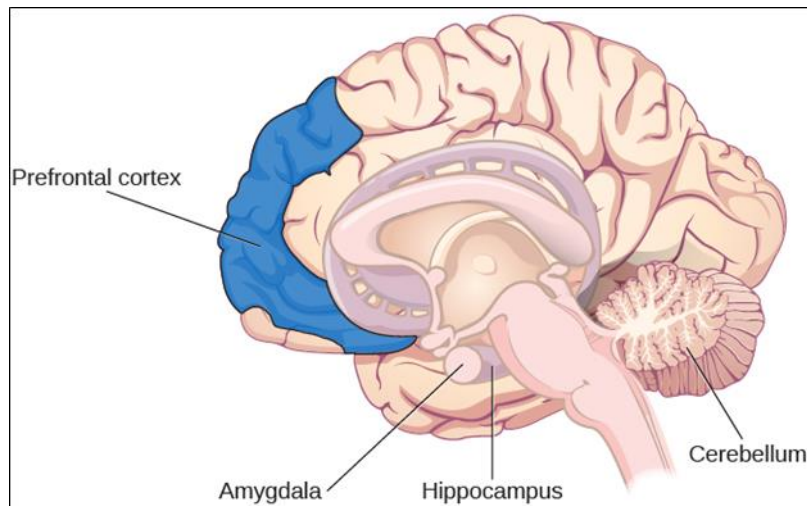


Figure 2-11 - Visual representation of the prefrontal cortex, and its association with the amygdala, hippocampus and cerebellum
(Adapted from <http://capgras.houston-psychologist.com>)

2.4.2 The Hippocampus

The hippocampus is an integral brain area involved in learning and declarative long-term memory (Eichenbaum and Cohen, 2001) and consists of two folded neuronal layers, i.e. the cornu ammonis (CA) and dentate gyrus (DG; see Figure 2-12). Functionally, the hippocampus mediates the relay between the initial realization of memories via primary cortical inputs and its final consolidation in other brain regions (Eichenbaum, 2000). Furthermore, the hippocampus moderates the stress response by preventing an inflated response of the HPA-axis during and following trauma. For example, the stress response prepares the body for the 'fight or flight' response, and enhances the memory of potentially life-threatening events, thereby optimising physiological and emotional responses to similar future events (McEwen, 2000; McEwen, 2007). Normally, successful adaptation results in reinstatement of the homeostatic balance. However, failure to cope with traumatic stress results in an altered equilibrium with significant neurobiological consequences. A number of neuroimaging studies have demonstrated hippocampal volume reductions in PTSD patients of both sexes (Bonne *et al.*, 2001) and that the extent of such reductions correlate with illness severity and the degree of cognitive deficit (Bremner, 1999). Moreover, such structural changes translate to poor cognitive performance in tasks dependent on normal hippocampal functioning (Samuelson, 2011). An important etiological role for hippocampal dysfunction in PTSD has been revealed in monozygotic twin studies that demonstrated hippocampal abnormalities to precede the onset of the disorder (Gilbertson *et al.*, 2002). These data indicate that the smaller hippocampi observed in PTSD represent a pre-existing familial vulnerability factor rather than being the product of neurotoxicity post-trauma exposure (Gilbertson *et al.*, 2002).

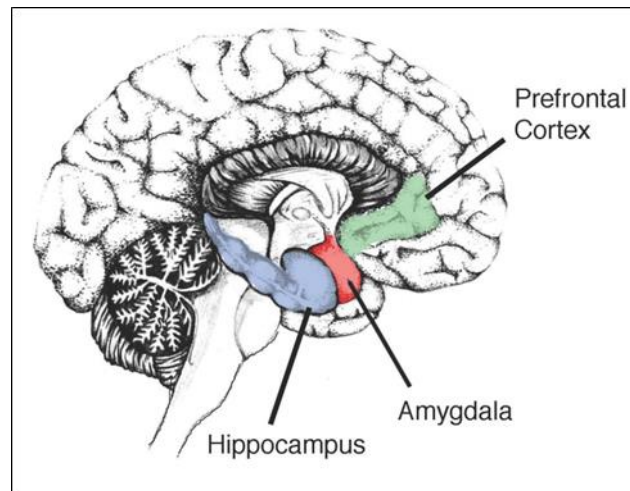


Figure 2-12 - Visual representation of the hippocampus, indicating its proximity with the PFC and amygdala and hippocampus (Adapted from <http://capgras.houston-psychologist.com>)

A link between hippocampal involvements and the aberrant HPA-axis responses in PTSD are evinced by the large number of gluco- and mineralocorticoid receptors found in the hippocampus that normally auto-regulate the HPA-axis via glucocorticoid-induced negative feedback (see Figure 2-13). However, the hippocampal damage observed in PTSD patients disrupts this negative feedback system, resulting in exposure to supra-physiological levels of glucocorticoids (Sapolsky, 2000) contributing to hippocampal atrophy (Laplante *et al.*, 2005). As such, hippocampal damage results in dysfunctional memory recall and inaccurate appraisals of potentially harmful situations that ultimately cause hyperarousal and exaggerated responses to cues of potentially traumatic circumstances (Laplante *et al.*, 2005).

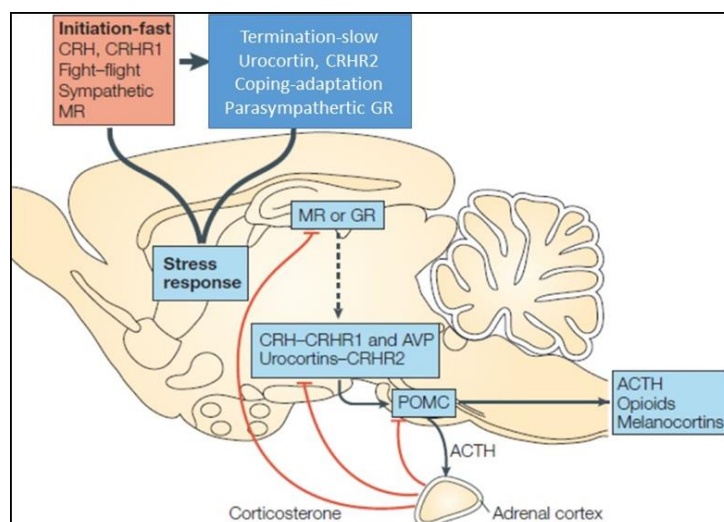


Figure 2-13 - The brain as an inducer and target of corticosteroids

Corticosteroids operate in both stress-system modes through mineralocorticoid and glucocorticoid receptors (MRs and GRs, respectively), which are co-expressed abundantly in the neurons of the limbic structures. (De Kloet *et al.*, 2005)

Animal models of PTSD, i.e. predator exposure (Cohen *et al.*, 2006a) and stress-restress (Harvey *et al.*, 2003a; Harvey *et al.*, 2006; Oosthuizen *et al.*, 2005b) have described a blunted HPA-axis response in trauma-exposed animals, resulting in an increased susceptibility to develop PTSD like symptoms. Therefore, sustained exposure to stressful events or reminders of traumatic experience may induce changes in HPA-axis responsivity that ultimately results in pronounced neuronal degeneration and cognitive impairment (Pynoos *et al.*, 1999). Of importance within the context of this study is that a suppressed cortisol response is associated with compromised stress coping mechanisms, thereby linking altered circadian cortisol release to the development of an anxiety and/or stress-related disorders (Dedovic & Ngiam, 2015).

2.4.3 The amygdala

Structural abnormalities of the amygdala in patients with PTSD have also been noted, with left amygdalar volumes being significantly smaller in patients with PTSD (Shin *et al.*, 2004; Karl *et al.*, 2006; Rogers *et al.*, 2009). 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). Recent investigations suggest that the amygdala, in association with the hippocampus, play a central role in the learning of fear responses and the processing of other basic emotional responses (Phelps *et al.*, 2004; Ledoux *et al.*, 2009). Whereas the hippocampus is responsible for the consolidation and recall of memory, the amygdala provides emotional significance to contextual stimuli (Phelps *et al.*, 2004; Ledoux *et al.*, 2009). Sensory inputs that trigger fear are relayed to the thalamus and are then routed *via* a fast-direct pathway to the amygdala that is responsible for its emotional processing, and a slow indirect pathway to the cortex that rationally and functionally appraises the situation (McGaugh & Roozendaal, 2002; see Figure 2-14). In turn, the amygdala responds to emotional stimuli with projections to the hypothalamus, activating the CNS (McGaugh & Roozendaal, 2002). Studies of brain functioning during traumatic exposure revealed hyperactive functioning of the amygdala in patients with PTSD (Shin *et al.*, 2004) that can be ascribed to its role in the consolidation and retrieval of aversive memories (Bremner *et al.*, 2007). Furthermore, the emotional stress and fear response results in the secretion of CRF by the amygdala that potentiates this autonomic reaction to fear (Liberzon *et al.*, 1999). Considering its role in emotional fear processing, the amygdala also plays an important role in the development of conditioned fear responses (Killcross & Place, 2000). As the amygdala is sensitive to inhibitory inputs from the prefrontal cortical dopaminergic system, it is evident that dopaminergic

disinhibition resulting from the cortical dysfunction as explained above in section 2.4.1, can contribute to the exacerbation of symptoms (Rauch *et al.*, 2003).

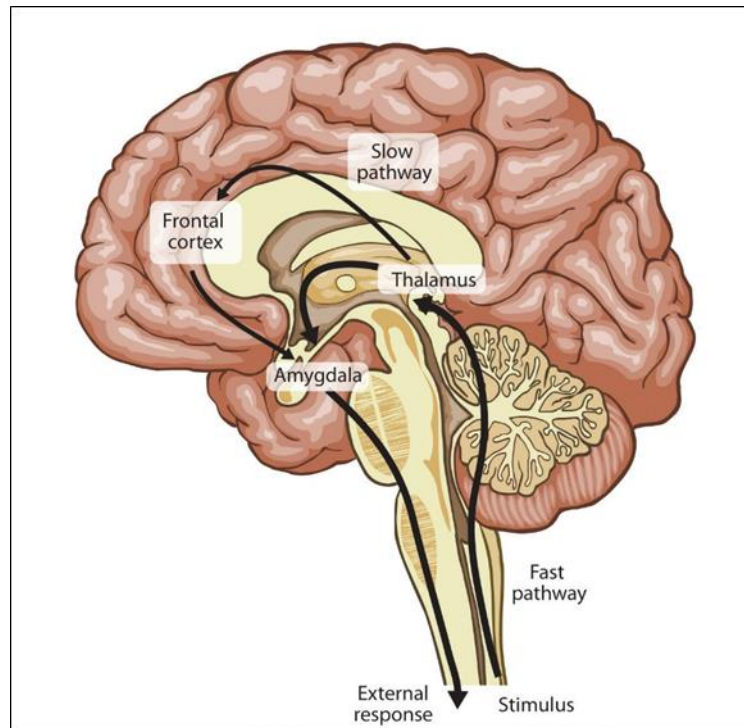


Figure 2-14 - The emotional pathways of the brain

There are two emotional pathways in the brain (one slow and one fast), both of which are controlled by the thalamus and directed to the amygdala that is responsible for its emotional processing (Stangor & Walinga, 2010)

2.5 The cognitive processes underlying PTSD

As discussed above, several structural and functional changes in the hippocampus, amygdala and prefrontal cortex have been found in PTSD (summarised in Figure 2-15). Due to the important role of these brain regions in the fear or stress response, emotion and cognition, it is however not surprising that such changes have clinical consequences. A recently proposed neurocircuitry model of PTSD suggests that hyper-responsivity of the amygdala may possibly underlie exaggerated fear conditioning (see sections 2.4.3) and explains the symptoms of hyperarousal as well as sustained or poor extinction of emotional memory of the trauma. Moreover, the model suggests that inadequate inhibition of the amygdala by the prefrontal cortex may explain deficits in extinction and attention, and mediate persistent recollection of traumatic memories and deficits in working memory. Finally, the model suggests that decreased hippocampal function may underlie deficits in identifying safe stimuli and mediate generalisation and cross-sensitisation of environmental stimuli (refer to section 2.4.2), as well

as dysfunction of explicit memory (Bonne *et al.*, 2004; Liberzon & Martis, 2006; Rauch *et al.*, 2006).

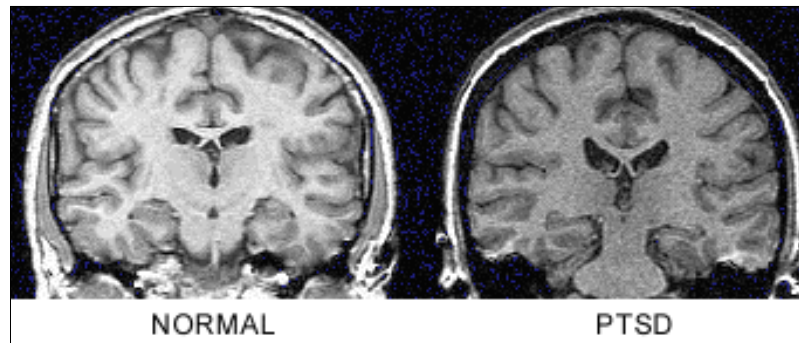


Figure 2-15 - MRI of a healthy individual vs. PTSD patient

Hippocampal volume reduction area involved in memory and increased activation of the amygdala area involved in emotional response (Bremner, 2000)

The primary symptoms of PTSD as mentioned earlier are re-experiencing, avoidance, numbing and hyperarousal, all related to neuronal mechanisms involved in fear and memory (Charney, 2004). Memory is conceptually organised into explicit and implicit components. The explicit component of memory includes verbal recall and working memory while implicit components include the fixed knowledge which is evident during the performance of learned tasks (Bremner, 1999). The cortical regions (prefrontal, anterior cingulate, and orbitofrontal) are all part of the neuroanatomical loci of memory processing. PTSD is characterised by a cognitive paradox, with disturbances in both explicit and implicit components of memory. This paradox is evinced by patients presenting with a remarkably strong memory of the traumatic event, i.e. fear memory (implicit memory), while at the same time experiencing amnesia for certain con-specific aspects of the traumatic event (explicit memory), i.e. people, place, and time. Patients suffering from PTSD display impairment in the ability to store and retrieve new information (Van Praag, 2004) while also presenting with increased formation of fearful memories related to the event that are resistant to extinction over time. The latter process sensitises the individual to trauma cues, thereby increasing the general alertness to potentially harmful stimuli (Siegmund & Wotjak, 2006). Fear conditioning (section 2.5.1 below), bolstered consolidation, failure of extinction, and subsequent sensitization (section 2.5.2 below), as well as learned helplessness and cognitive processing deficits have all been proposed to contribute to the three core symptoms of PTSD (Bonne *et al.*, 2004; Charney, 2004).

2.5.1 Fear conditioning

As alluded earlier, autonomic arousal, vivid memories, and even flashbacks observed in PTSD can be activated by both internal and external stimuli (Bonne *et al.*, 2004). The emotional response that follows is extremely stressful and as a result, numbing of general emotional responsiveness may occur as patients try to avoid these stimuli (Charney, 2004). Fear conditioning is a psychobiological mechanism that may account for the association between traumatic re-experiencing symptoms and harmless stimuli (Charney, 2004). In an animal, when a non-threatening stimulus is presented together with an aversive stimulus, the animal later exhibits a fear response also to the appearance of the non-threatening stimulus alone (Bonne *et al.*, 2004). Thus, fear conditioning is a positive adaptive mechanism in life-threatening situations, enhancing an animal's response and attention to danger (Sanders *et al.*, 2003; Charney, 2004). In contrast, fear conditioned responses in PTSD are maladaptive and may represent a loss of stimulus perception, i.e. separating true vs. unlikely danger. Individuals suffering from the disorder may therefore display re-experiencing and hyperarousal symptoms as a result of both threat- and non-threat related stimuli (Bonne *et al.*, 2004). Fear conditioning studies in patients with PTSD show increased heart rate and skin conductance to the conditioned stimulus compared to trauma-exposed controls (Orr *et al.*, 2000). Fear conditioning, involving glutamatergic signalling *via* ionotropic AMPA and NMDA, and metabotropic mGluRs, is critically involved in learning and memory (Walker *et al.*, 2002; Walker *et al.*, 2003; Nihei *et al.*, 2000). Since fear extinction replicates the process of an active inhibitory process, it is possible that GABA, the major inhibitory neurotransmitter in the brain, serves as the source of that inhibition *via* its actions at ionotropic (GABA_A) and metabotropic (GABA_B) receptors. However, it has been disputed for some time that GABA is involved in the consolidation of excitatory learning, as GABA agonists disrupt (Davis & Myers, 2002; Bermudo-Soriano *et al.*, 2012; Averill *et al.*, 2017) and GABA antagonists facilitate (Davis & Myers, 2002; Bermudo-Soriano *et al.*, 2012) acquisition of aversively motivated tasks. Therefore, therapies targeting fear conditioning and its associated neurobiological processes may be clinically useful in the treatment of stress-induced disorders. As such, drawing from above, the NMDA receptor antagonist, memantine, may be clinically useful in the treatment of stress-induced disorders (Charney, 2004). Further, it has been found that the partial agonist at the glycine regulatory site on the NMDA receptor, cycloserine, has proved clinical efficacy in treating PTSD (Heresco-Levy *et al.*, 2002) and adds incentive to the possible pathological role of the glutamate NMDA receptor in the disorder. Another approach may

be to reduce the impact and intensity of recently acquired fear memories with clinically available Ca^{2+} channel blockers, e.g. verapamil and nimodipine (Charney, 2004); this since glutamatergic NMDA receptors in the hippocampus mobilize a number of intracellular messengers such as Ca^{2+} that are ultimately responsible for the neuronal and behavioural effects induced by NMDA receptor activation (Harvey *et al.*, 2005b). These consist of critical Ca^{2+} dependent processes such as activation of neuronal nitric oxide (NO) synthase (nNOS) and the release of NO (Harvey *et al.*, 2005b), activation of the soluble guanylate cyclase and release of cyclic guanosine monophosphate (cGMP), and the activation of the nuclear transcription factor, $\text{NFK}\beta$ and inducible NOS (iNOS; Burr & Morris, 2002; Lee *et al.*, 2006). Indeed, preclinical work has confirmed a role for iNOS (Madrigal *et al.*, 2001), $\text{NFK}\beta$ (Madrigal *et al.*, 2001), glutamate (Harvey *et al.*, 2004b), iNOS (Corrêa *et al.*, 2007), and cGMP (Volk *et al.*, 1997; Volke *et al.*, 2003) in numerous models of anxiety and stress.

2.5.2 Fear memory reconsolidation, extinction, and sensitization

The strength and regulation of fearful memories is exaggerated by numerous factors both before and after the traumatic or fearful event occurs. Genetic heritability comprises up to 30 – 40% of the risk for both depression and PTSD, and early childhood mishandling is a strong risk factor for all mental disorders including PTSD (Zeanah & Gleason, 2010). Memories are not steady at the time of the trauma, and pharmacological methods as well as psychological methods to prevent the early encoding of the trauma are promising but still unconfirmed (Zeanah & Gleason, 2010).

Memories tolerate a period of consolidation in which they change from a labile to a fixed state (Caithness *et al.*, 2004). It can therefore be considered that impairing the consolidation process would be an alternative way to prevent the formation of long-term trauma memories. The appearance of stressful memories, which can be the cause of symptoms in fear-related disorders such as PTSD, is weakened by the sequence of extinction, a process whereby repeated exposure to specific fear-related cues inhibit fear memories over time (Jovanovic & Ressler, 2010). Briefly, when recognizable signals for aversive events no longer predict actual trauma, the previously learned conditioned emotional fear response is reduced and eventually disappears (Maren & Quirk, 2004). Extinction therefore may involve the learning of new associations that compete with, or override the original, fear response producing association (Chambers *et al.*, 1999; Anderson & Insel, 2006). However, there is some evidence that in individuals who develop PTSD and other anxiety disorders, a combination of avoidance of

sufficient exposure with unpleasant and intense memories leads to sensitization of the fear response. In addition, the ability of conditioned fear to be reinstated may explain the clinical observation that dormant traumatic memories may be triggered by a subsequent stressor or by a stimulus related to the original trauma (Sanchez & Sorg, 2001). Moreover, the leading vulnerability factor for PTSD is past history of trauma and childhood abuse, suggesting that re-exposure to a traumatic event even after extinction of the original fear response, could trigger PTSD (Bonne *et al.*, 2004). Moreover, constant activation of traumatic memories may strengthen such memories so that with each episode of recall, it becomes part of a new memory. Therefore, bolstered reconsolidation is central to PTSD and has been proposed to contribute to persistent trauma-related symptoms (Bonne *et al.*, 2004; Charney, 2004). Improving the perception and extinction of fear memories is a vital aspect of recovery in the psychotherapeutic methods applied in the treatment of PTSD (Jovanovic & Ressler, 2010; see Figure 2-16).

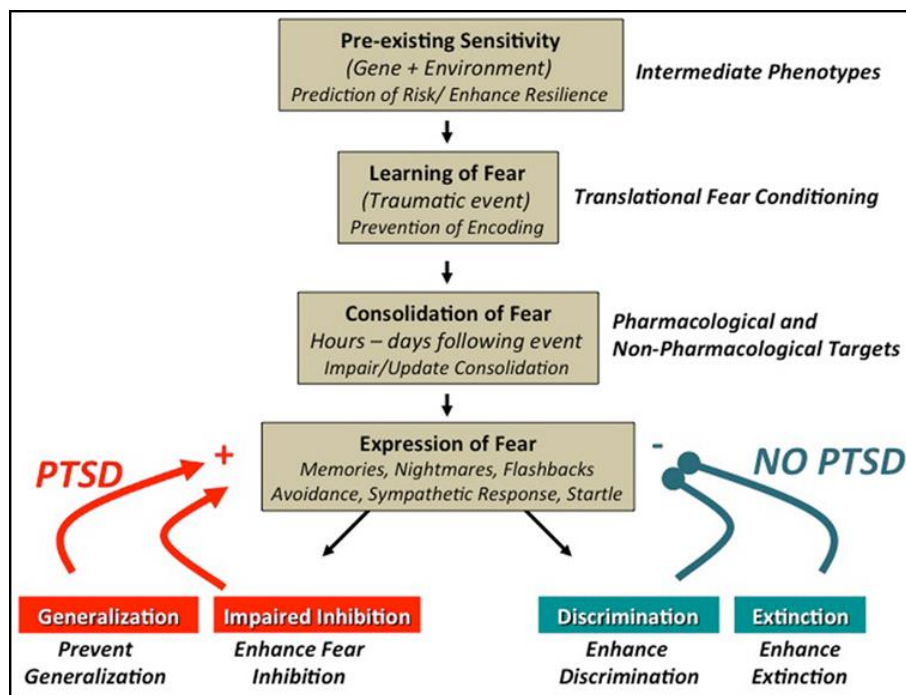


Figure 2-16 - Schematic diagram representing the developmental progression of PTSD vs. no PTSD with regards to consolidation of fear and extinction (Briscone *et al.*, 2014)

2.6 The treatment of PTSD

Current treatments for PTSD include various classes of antidepressants and anxiolytics, although noradrenergic modulators as well as modulators of the HPA-axis have also been investigated. Furthermore, the condition also demonstrates response to psychotherapy. Section 2.6 will provide a broad overview of the current treatment strategies employed in the management of PTSD.

2.6.1 Antidepressants

Antidepressants have been found to be effective not only in major depressive disorder, but also in other psychiatric conditions e.g. panic disorders, obsessive compulsive disorder, chronic pain, and eating disorders (Borsini *et al.*, 2002). Given the high degree of comorbidity between PTSD and depression and considering the symptomological similarity between PTSD and other anxiety disorders, e.g. agoraphobia and panic attacks, early research has investigated the efficacy of antidepressants for the treatment of PTSD. Indeed, various classes of antidepressants, i.e. SSRIs, SNRIs, and MAOIs, are employed in the treatment of the non-cognitive symptoms of PTSD (Borsini *et al.*, 2002). Broadly stated, their actions are based on bolstering synaptic monoamine levels that are involved in modulating arousal, mood, stress responses, and anxiety (McEwen, 2000). Consequently, it was thought that elevating levels of catecholamines and serotonin may correct abnormalities in the central nervous system altered by trauma. Now researchers are looking at secondary adaptive mechanisms as an explanation of their efficacy. Indeed, many PTSD symptoms, i.e. enhanced fear, anger, arousal, and aggression, all suggest dysregulation of one or more of the monoamine transmitters (Yehuda *et al.*, 1992) that constitute the neurobiological rationale for the use of these agents in PTSD (Newport & Nemeroff, 2000).

SSRIs, e.g. sertraline and fluoxetine, were the first drugs approved for the treatment of PTSD (Vaswani *et al.*, 2003). SSRIs selectively block the reuptake of synaptic 5HT by modulating the 5HT transporter (Vaswani *et al.*, 2003). SSRIs are well tolerated and have a relatively benign side effect profile, with gastrointestinal complications and sexual dysfunction being most commonly reported (Albucher & Liberzon, 2002; Vaswani *et al.*, 2003). Several open and controlled trials indicate that SSRIs are effective in improving PTSD symptoms in at least two of the symptom clusters, viz. arousal and avoidance/numbing but not for the third symptom cluster, re-experiencing/intrusion (Marshall *et al.*, 2001; Spivak *et al.*, 2006). SNRIs e.g. venlafaxine, desvenlafaxine, duloxetine, and milnacipran bind to norepinephrine and 5-HT

transporters and inhibit the reuptake of NA and 5HT from the synaptic cleft (Dell'Osso *et al.*, 2010). As SNRIs have the advantage of acting on different areas of monoamine functioning simultaneously, they may address different neurobiological constructs. For example, while aggression has been ascribed to 5HTergic mechanisms (Healy & McMonagle, 1997), deficits in motivation are often associated with altered noradrenergic signalling (Healy & McMonagle, 1997).

Although not approved for the treatment of PTSD, tricyclic antidepressants (TCAs) are occasionally considered as an alternative treatment (Asnis *et al.*, 2004). The TCAs are catecholamine/indolamine reuptake blockers, with varying degrees of noradrenalin (NA) and 5HT reuptake inhibition depending on the drug (Asnis *et al.*, 2004). This group also antagonises α_2 -adrenergic-, histaminergic and cholinergic muscarinic receptors, resulting in a broad side-effect profile that includes sedative and cardiac effects (Asnis *et al.*, 2004). Controlled trials (Davidson *et al.*, 1990; Kosten *et al.*, 1991; Briere & Scott, 2014) and open-label trials as well as several case studies (Burstein, 1984; Falcon *et al.*, 1985; Stein *et al.*, 2006; Davis *et al.*, 2016) demonstrate efficacy of imipramine and amitriptyline, but not desipramine in global improvement of PTSD. Despite higher failure rates due to poor tolerability, TCAs like imipramine and amitriptyline should therefore be considered as valid alternatives in patients who are intolerant to SSRIs (Albucher & Liberzon, 2002). Of note is that in all of the controlled- and most of the uncontrolled trials for TCAs, the participants were combat veterans suffering from chronic PTSD. Their modest efficacy in this notoriously treatment resistant population should therefore be given serious consideration (Albucher & Liberzon, 2002).

The mechanism of action of the monoamine oxidase inhibitors (MAOIs) involves inhibition of the enzyme monoamine oxidase (MAO) and consequently, the potentiation of serotonergic, dopaminergic and noradrenergic neurotransmission (Asnis *et al.*, 2004). The MAOIs also block histaminergic- and α_2 -adrenoceptors, which might contribute to their adverse effects (Asnis *et al.*, 2004). Interest in the possible role of MAOIs in the treatment of PTSD has led to various case reports and open-label trials (Davidson *et al.*, 1987; Neal *et al.*, 1997). Several controlled studies have also been undertaken, but their results are inconsistent (Shestatzky *et al.*, 1988; Kosten *et al.*, 1991; Katz *et al.*, 1994; Baker *et al.*, 1995). Regardless of these mixed findings, PTSD patients treated with this class of drug show greater global improvement than those treated with TCAs (Albucher & Liberzon, 2002). Although dietary restrictions when

using MAOIs, risk of hypertensive crises, and high non-adherence rates restrict its clinical usefulness (Albucher & Liberzon, 2002), MAOIs remain under reserved use for treatment-resistant cases (Albucher & Liberzon, 2002).

2.6.2 GABA and glutamate modulators

GABA has an important role in attenuating excessive glutamatergic activity in the brain and in modulating activation of the stress response. Suppression of GABA levels following severe trauma results in gradual disinhibition of glutamatergic activity (Oosthuizen *et al.*, 2005a); indeed, either bolstering GABAergic neurotransmission and/or inhibiting glutamatergic signalling have been demonstrated to be an effective approach to treating PTSD (Lydiard, 2003). By virtue of their clinical efficacy in decreasing arousal and anxiety and in promoting sleep, as well as deactivating the amygdala (Del Ben *et al.*, 2012), there has been some interest in using the benzodiazepines, e.g. alprazolam and clonazepam in the treatment of PTSD. Acting as agonists at the GABA_A receptor (Borchardt, 1999), benzodiazepines potentiate the actions of GABA. However, despite the said promise from a mechanistic point of view, benzodiazepines are ineffective in the treatment of PTSD and may in fact worsen the condition (Mellman *et al.*, 2004). This appears to be a direct consequence of amplifying GABA transmission (Gelpin *et al.*, 1996). The underlying mechanism requires deeper study, although may be due to the broad non-specific effects of GABA inhibition on a number of other key transmitters systems involved in fear responding and extinction. Moreover, clinical observations include the likelihood of dependence, while early discontinuation may worsen PTSD symptoms after withdrawal as well as increase the incidence of PTSD after early treatment (Ursano *et al.*, 2004). Thus, benzodiazepines cannot be recommended as monotherapy in PTSD (Ursano *et al.*, 2004). On the other hand, it has been demonstrated that non-benzodiazepines may be useful in PTSD via their indirect GABAergic actions (Garner *et al.*, 2009). For instance, topiramate and valproate increase the synthesis of GABA, whereas vigabatrine inhibits its breakdown (Khan & Liberzon, 2004); these drugs have been found effective to varying extent in treating clinical PTSD (Khan & Liberzon, 2004). Further, GABA analogues, i.e. pregabalin and gabapentin, can also be useful (Garner *et al.*, 2009).

Given that both fear conditioning and extinction are dependent on glutamatergic NMDA receptor signalling in the amygdala (Walker *et al.*, 2002), it is not surprising that treatment of PTSD with the partial NMDA receptor agonist, D-cycloserine, has been found effective in improving numbing, avoidance, and anxiety in patients with PTSD (Heresco-Levy *et al.*, 2002).

Moreover, these findings have broadly been reproduced in various animal studies (Khan & Liberzon, 2004; Harvey *et al.*, 2005a). Considering the anxiolytic response of buspirone, a partial 5HT_{1A}-receptor agonist (Argyropoulos *et al.*, 2000), a number of clinical reports indicated that it may be a safe and effective alternative treatment for PTSD (Duffy & Malloy, 1994; Fichtner & Crayton, 1994; Stein *et al.*, 2006).

2.6.3 HPA-modulators

Ketoconazole is an imidazole antimycotic agent that potently inhibits several cytochrome P450-dependent enzymes involved in gonadal and adrenal steroidogenesis and has demonstrable clinical efficacy in the treatment of PTSD (Cohen *et al.*, 2000). Ketoconazole inhibits the synthesis of ergosterol in fungi and cholesterol in mammalian cells by blocking 14-demethylation of lanosterol (Cohen *et al.*, 2000). It is also effective as a palliative treatment for Cushing's syndrome due to its ability to lower cortisol production. High dose (24 mg/kg) acutely administered ketoconazole significantly increased plasma ACTH levels in normal rats (Burrin *et al.*, 1986) and in man (Cohen *et al.*, 2000) confirming that high dose ketoconazole therapy inhibits adrenal steroid production. The fall in corticosterone levels removes negative feedback to the hypothalamus pituitary, thereby stimulating an increase in ACTH secretion. It has been reported that an adaptation to chronic stress may occur, resulting in decreased plasma ACTH and corticosterone levels compared with levels following a single stressor. Since this approach had been successfully applied in the treatment of depression (Lamberts *et al.*, 1997; Wolkowitz *et al.*, 1999), ketoconazole has subsequently been proposed as a treatment for chronic comorbid PTSD and depression (Cohen *et al.*, 2000). Investigations done in humans with PTSD (Pitman & Orr, 1990; Yehuda *et al.*, 1990; Yehuda *et al.*, 1991; Yehuda & Antelman, 1993; Yehuda, 1997) have however demonstrated conflicting results, although current opinion inclines to the view that cortisol is reduced in PTSD (Pitman & Orr, 1990).

Another strategy for preventative treatment that are in line with aberrant cortisol responses would be to improve stress coping, i.e., to facilitate stress resilience. A number of preclinical studies have investigated molecular mechanisms involved in the stress response, one being a focus on the CRF systems. CRF₁ receptor antagonism has been found to prevent the initiation of stress effects in a mouse predator stress model of PTSD (Adamec *et al.*, 2010). These effects may be mediated *via* central effects at limbic circuitry or *via* inhibition of the HPA-axis. CRF₁ receptor antagonism in the amygdala attenuates fear conditioning (Roosendaal *et al.*,

2002; Hubbard *et al.*, 2007), which may contribute to the effects of these drugs. For drugs affecting HPA-axis activity however, these findings would suggest that interventions preventing an exaggerated stress response may be favourable prior to the occurrence of the traumatic event. Following this line of interpretation, it would also be possible that other classes of compounds that block HPA activity, such as vasopressin V_{1b} antagonists, could offer therapeutic utility. Interestingly, vasopressin has been shown to affect the memory consolidation processes, either directly or indirectly further strengthening this case (Griebel *et al.*, 2002). Several studies have demonstrated increased plasma levels of vasopressin in anxiety disorders as well as in unipolar depression (De Kloet *et al.*, 2008). Vasopressin is released in the hypothalamus, pituitary, and limbic regions and contributes to the endocrine and neuronal responses to stress (Surget & Belzung, 2008). Antagonism of the vasopressin V_{1b} receptor has been shown to decrease anxiety and depressive-like behaviours in rodents, and to attenuate the HPA-axis response to stress. Therefore, certain affective disorders may be related to excessive vasopressin functioning (Surget & Belzung, 2008). In line with this hypothesis, the selective V_{1b} receptor antagonist, SSR149415 (Griebel *et al.*, 2002; Serradeil-Le Gal *et al.*, 2002) inhibits restraint stress induced ACTH secretion in rats (Serradeil-Le Gal *et al.*, 2002), while blockade of pituitary (Shimazaki *et al.* 2006) and amygdalar (Salomé *et al.* 2006) V_{1b} receptors results in anxiolytic-like effects. It can therefore be postulated that V_{1b} receptor antagonists may represent a promising alternative to agents currently used for the treatment of PTSD (Griebel *et al.*, 2005). Considering the other posterior pituitary hormone, oxytocin, it has been shown to attenuate glucocorticoid secretion (Windle *et al.*, 1997) and to elicit anxiolytic-like effects in animals (Heinrichs *et al.*, 2003) and humans (Legros, 2001; Heinrichs *et al.*, 2003). Oxytocin has therefore been proposed as a putative anxiolytic (Windle *et al.*, 1997) with application in PTSD (Koch *et al.*, 2014).

2.6.4 NA-modulators

Previously, the $\beta_{1/2}$ adrenoceptor antagonist, propranolol, was found to relieve increased startle response, nightmares and intrusive re-experiencing in some patients with PTSD (Friedman, 1997), while the α_1 -adrenoceptor antagonist prazosin, a commonly used anti-hypertensive agent, has shown promise in treating sleep disturbances in patients with chronic PTSD (Taylor & Raskind, 2002). Both drugs inhibit excessive noradrenergic activity that is associated with fear and startle responses in PTSD that normally result in heightened emotional arousal. Other anti-adrenergic drugs tested in PTSD include the α_2 -adrenergic

agonists, clonidine and guanfacine. Clonidine and guanfacine have been shown to effectively reduce PTSD symptoms such as nightmares, insomnia, intrusive recollections, startle reactions and hypervigilance in patients with PTSD (Charney *et al.*, 1992; Harmon & Riggs, 1996; Horrigan & Barnhill, 1996; Boehnlein & Kinzie, 2007). However, their clinical application is not widespread since α_2 AR-mediated regulation of CNS function extends to the peripheral nervous system as well. Still, literature increasingly points to selectively targeting specific α_2 AR subtypes to apply control over presynaptic modulation of various neurotransmitter feedback systems related to cognitive and affective functioning, since work on non-selective α_2 antagonists have shown that both α_{2A} and α_{2C} -receptors inhibit the release of NA (Hein *et al.* 1999); yet, the α_{2C} -receptor system is less prominently involved in presynaptic inhibition compared to the α_{2A} -receptor (Bücheler *et al.* 2002). That said, the potency of NA at the α_{2C} AR is higher than it is at the α_{2A} AR, which correlates with the difference in affinity of NA for the two receptors (Hein *et al.* 1999). These qualities are of importance especially when considering selective α_{2C} AR antagonists in animal models characterised by hyper adrenergic states where symptoms have been attributed to noradrenergic over activity (Yamamoto, Hornykiewicz 2004).

2.6.5 Psychotherapy

Psychotherapy is often employed to address aberrant cognition underlying mental illness. In this regard, the clinical practice guidelines developed by the International Society for Traumatic Stress Studies (Foa *et al.*, 2008), suggest that exposure and response prevention (ERP) therapy is the most robust and successful psychotherapeutic intervention for PTSD. However, cognitive behavioural therapy (CBT; Resick *et al.*, 2002) and interpersonal psychotherapy (Bleiberg & Markowitz, 2005) are also effective. Furthermore, eye movement desensitization and reprocessing (EMDR; Shapiro & Solomon, 1995), which involves accessing traumatic images and memories, evaluating its aversive qualities, and focusing on alternative cognitive appraisals (Nemeroff *et al.*, 2006), is also advocated. These approaches seek to focus on the patients' memories of their traumatic events and the personal meanings of the trauma (Ehlers *et al.*, 2010).

2.7 Animal Models of PTSD

2.7.1 Designing animal models of PTSD

In the attempt to emulate psychiatric disorders in animal models, three principles of validity need to be met, viz. face-, construct- and predictive validity (Overstreet, 1993). They can be summarized and defined as follows:

- Face validity: Similarity of behavioural dysfunction, i.e. symptoms, that can be seen in both the animal model and the human disorder;
- Construct validity: Similarities to the human disorder with respect to the mechanisms that underlie the disordered behaviour evident in the model; and
- Predictive validity: The ability of established drug treatments used in the human illness to show comparable efficacy in the animal model in line with clinical response, while drugs that are ineffective in the human condition are also ineffective in the animal.

Furthermore, with respect to PTSD, certain criteria have been set in order to validate, assess and apply animal models of PTSD (Yehuda & Antelman, 1993), viz.:

- Even very brief stressors should be capable of inducing biological and behavioural sequelae of PTSD. A stressor is often measured by its severity and duration, but the defining factor in an animal model should rather be the ability of the stressor to elicit bio-behavioural changes associated with the PTSD symptomatology.
- The stressor should be capable of producing the PTSD-like sequelae in a severity dependent manner. This should be achieved without making alterations to the duration of the stressor as this parameter has little relevance in the clinical situation of PTSD.
- The stressor should produce biological alterations that persist and become more pronounced over time. Given that the onset of PTSD following exposure to trauma is often delayed for months or even years, an animal model should present with a gradual worsening of biological abnormalities over time. Studies of animal models of PTSD would have to include long term assessments in addition to identifying short-term sequelae of stress. Further, an animal model should also allow for the possibility of recovery from short-term symptoms since it has been found that not all subjects go on to develop the pathological condition.

- The stressor should induce bio-behavioural alterations that have the potential for bi-directional expression. The core symptoms of PTSD include both enhanced (intrusive re-experiencing) and reduced (avoidance and/or numbing) responsiveness to stimuli. Although avoidance-related symptoms are thought to be one of the earliest manifestations following a traumatic event, other symptoms with a delayed onset, e.g. intrusive memories, imply that not all symptoms are simultaneously exhibited. Therefore, symptom clusters must be analysed taking this into account. However, the inherent capacity of animal models to emulate this bio-directionality is often compromised.
- Inter-individual variability with respect to response to stressors should be present either as a function of experience (e.g. prior stress history and post-stressor adaptations) and genetics, or an interaction of the two. Although trauma is the most common cause of PTSD, it is not solely responsible for triggering PTSD. This criterion is concluded from the fact that not all individuals who are exposed to the same trauma will develop PTSD.

As such, a number of robust and useful animal models have been established based on the abovementioned criteria. They include the inescapable shock-learned helplessness (IS-LH) model (Bonne *et al.*, 2004), the time dependent sensitization (TDS) model (Liberzon *et al.*, 1997; Harvey *et al.*, 2003a; Harvey *et al.*, 2004a; Harvey *et al.*, 2004b; Harvey *et al.*, 2006), the fear-potentiated startle model (Morgan *et al.*, 1995), and the predator stress model (Adamec & Shallow, 1993). The following paragraph summarizes a number of the models that are especially relevant for the current investigation.

2.7.1.1 The predator scent exposure model (PSEM)

The PSEM has ethological relevance as it mimics intense stressful experiences and results in long term changes in behavioural, autonomic and hormonal responses that correlate with the symptoms in human PTSD (Cohen *et al.*, 2003). Regarding the conceptual validity of the model, “predator exposure trauma” is a potentially life-threatening situation and may represent a more “natural” challenge than other forms of stressors, i.e. electric tail shocks or restraint (Adamec *et al.*, 1997); these may in fact be more related to extreme conditions such as torture. Referring to similarity of symptoms in the animal model and PTSD (van der Staay *et al.*, 2009), the PSEM demonstrates hyperarousal (Hebb *et al.*, 2003; Cohen *et al.*, 2004; Cohen *et al.*, 2006a; Cohen *et al.*, 2008), increased anxiety (Adamec *et al.*, 2006; Cohen *et al.*,

2008; Muñoz-Abellán *et al.*, 2008), symptoms of social withdrawal (Zangrossi & File, 1992) as well as increased freezing and avoidance behaviour (Blanchard *et al.*, 1990a; Wallace & Rosen, 2000; Dielenberg *et al.*, 2001; Masini *et al.*, 2005), and ultrasonic vocalization (Roseboom *et al.*, 2007). In evaluating the similarities between the neurobiological mechanisms underlying aberrant behaviour in the human and animal model, the molecular and biological changes evident in the animal model correlate with that described in PTSD (van der Staay *et al.*, 2009). First, increased glutamatergic and noradrenergic signalling, as well as an increase in glucocorticoid and noradrenalin release (de Kloet *et al.*, 1999) have been described. Second, the model has proved valuable for studying HPA-axis abnormalities relevant to PTSD (Yehuda & Antelman, 1993), such as altered corticosterone concentrations, elevated sympathetic activity, diminished vagal tone, and altered sympathovagal balance (Cohen *et al.*, 2003). Lastly, animals subjected to predator exposure also present with enhanced sensitivity to negative glucocorticoid feedback that is often characteristic of PTSD (Cohen *et al.*, 2003). Concerning the response of the model to pharmacological interference, noradrenergic receptor antagonists, e.g. propranolol (Ferry *et al.*, 1999; Pitman *et al.*, 2002), steroid synthesis inhibitors (Ketoconazole) (Cohen *et al.*, 2000), chronic antidepressant treatment (Dielenberg & McGregor, 2001), as well as alcohol (Blanchard *et al.*, 1990b), attenuate stress-related behaviours in this model.

Predator prey relationships have been of notable interest in various studies (Apfelbach *et al.*, 2005). Prey species present with various adaptations that allow recognition and avoidance of, and defence against predators, i.e. sensitivity towards predator-derived odours (Apfelbach *et al.*, 2005). Predator odours can come from various sources such as predator skin, fur, urine, faeces and anal gland secretions (Apfelbach *et al.*, 2005). Laboratory as well as field studies have shown that predator odours exert distinct behavioural effects, i.e. 1) inhibition of investigative activity, 2) suppression of non-defensive behaviours such as foraging, feeding and grooming, and 3) changes in habitats or locations where such odours are absent (Blanchard *et al.*, 2005). Furthermore, it has been demonstrated that predator odours have influential effects on the endocrine system causing an increase in ACTH and corticosterone secretion (Blanchard *et al.*, 1993). Predator odours also have an effect on reproductive behaviour particularly prevalent in female rodent species, since females exposed to predator scent may give birth to smaller litters. A number of studies have presented evidence of the changes that occur in primary behavioural defences that assist in avoiding potential predators following the exposure to relevant predator odours (Blanchard *et al.*, 2003a). Acute exposure of rats to

predator odours has been demonstrated to cause significant and relatively long-term decreases in overall locomotor activity and non-defensive behaviours, e.g. grooming and reproduction, and to induce flight to strategic locations where the predator or predator odours can be detected, but avoided (McGregor *et al.*, 2002). A study done by Blanchard *et al.* (2003) showed that laboratory rats showed a very clear and distinctive avoidance response towards cat odour, introduced as a cloth rubbed on a cat, cat faeces, cat urine, or to TMT (2,3,5-trimethyl-3-thiazoline), a component of fox odour. Furthermore, the cat cloth was able to produce contextual conditioning of fear to the environment in which it was presented (Blanchard *et al.*, 2003a).

Odours of predators are received via two sensory organs, namely the main olfactory system with the olfactory epithelium located inside the nose, and the vomeronasal organ (VNO), which, in rodents, opens directly into the anterior nasal cavity (Halpern & Martinez-Marcos, 2003). The olfactory system detects different volatile odour molecules, while the VNO specifically is specialized to receive within species messages (pheromones) relating to reproduction, aggression and defence (Halpern & Martinez-Marcos, 2003). Odour molecules interact with receptor cells in either the VNO or in the olfactory epithelium where chemical messages of the molecules are encoded into electrical signals. These are then transmitted to the main olfactory bulb (MOB); the VNO sends its axons to a specialized region in the posterior part of the olfactory bulb, known as the accessory olfactory bulb (AOB) (Halpern & Martinez-Marcos, 2003). Each odour that is received results in a specific neural activity in the olfactory bulb that are transferred to the limbic and cortical regions for further processing (Halpern & Martinez-Marcos, 2003; see Figure 2-17).

Rats exposed to cat odours in confined environments demonstrate robust activation of the cell layers in the AOB but show little activation in the MOB. This implies that that predator odour is processed in the AOB as a pheromone, and not as an orthodox odour (McGregor *et al.*, 2004). This suggests cat odour to be an example of a “kairomone”, a semiochemical (pheromone or other chemical that conveys a signal from one organism to another so as to modify the behaviour of the recipient organism that is released by one species and that has a favourable (or detrimental in susceptible subjects) adaptive effect on a different species (Dicke & Grostal, 2001). Interestingly, synthetic fox odour, TMT, does not activate the AOB but causes forceful localized activation of the MOB (Staples *et al.*, 2008), explaining the differences in defensive responses to cat and fox odour. Fox urine has been shown to activate the MOB and piriform cortex as well as the basolateral and central nucleus of amygdala that is involved

in the fear response (Funk & Amir, 2000). However, the AOB projects predominantly to the medial amygdala that, following exposure cat odour, show substantial activation (Dielenberg *et al.*, 2001).

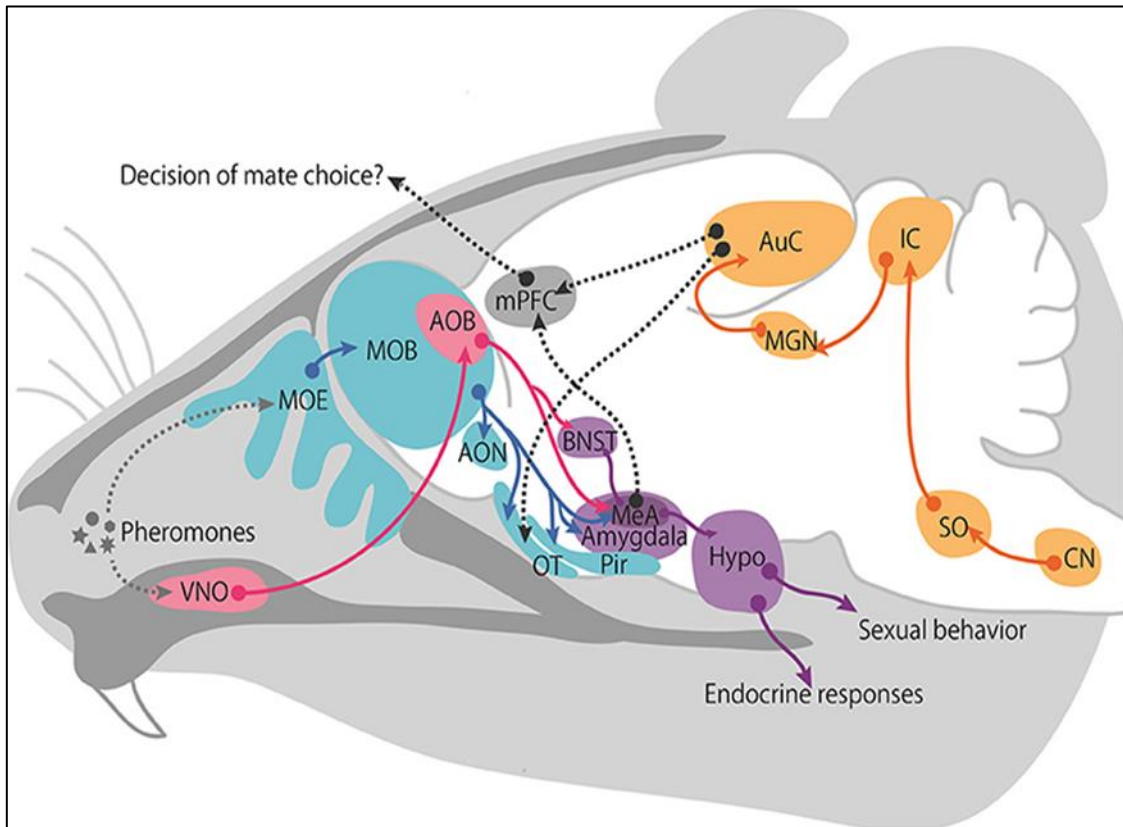


Figure2-17 - Schematic diagram of the olfactory physiology (Asaba *et al.*, 2014)

Other studies (Canteras, 2002; Blanchard *et al.*, 2003a; Blanchard *et al.*, 2003b) have demonstrated the medial hypothalamic circuit to play a vital role in establishing defensive responses to predators and predator odour (Blanchard *et al.*, 2003b). Therefore, behavioural changes provoked by predator odour may be instigated at a hypothalamic level and may rely on the well-recognised outflow from both the hypothalamus and periaqueductal grey matter (Canteras, 2002). This circuitry is important in inhibiting foraging and exploratory activity in response to predator or predator odours and in allowing prey to learn appropriate responses to predators (Staples & McGregor, 2006). Predator odours not only affect the nervous system directly, but also modify behaviour *via* actions on the endocrine system (Figueiredo *et al.*, 2003). Work done by File *et al.* (1993) demonstrated an increase in circulating corticosterone following exposure to cat odour, but that such increases were reversed following repeated exposure (Figueiredo *et al.*, 2003). With respect to the current investigation, Cohen *et al.* (2000) reported that when rats were exposed to cat odour, they

demonstrated anxiety-like behaviour 7 days later. These findings are not only in line with other PSEM studies (Adamec *et al.*, 1998; Dielenberg & McGregor, 1999) but also with other PTSD models such as stress-restress (Harvey *et al.*, 2006). Further, congruent with clinical literature, the gonadal and adrenal steroidogenesis inhibitor ketoconazole suppresses corticosterone, prolactin and ACTH levels induced by cat cloth exposure (Cohen *et al.*, 2000).

In conclusion, an abundance of literature confirms that small mammalian species that are chronically exposed to predator odours tend to present with altered foraging, feeding, general anxiety as well as reproduction in females (Apfelbach *et al.*, 2005). Predator odours therefore have a powerful stress-like effect on the endocrine system and cause an increase in corticosterone levels (Apfelbach *et al.*, 2005).

2.7.1.2 *Time-dependent sensitization (TDS) (stress-restress) paradigm*

The term "time-dependent sensitization" (TDS) refers to the fact that exposure to a stressor (e.g. restraint stress) will have sustained consequences for chronic periods of time, altering the animal's response to subsequent pharmacological or non-pharmacological stressors (Yehuda & Antelman, 1993). The model involves single exposure to a severe stressor with subsequent sensitization during re-stress to induce PTSD like symptoms (Liberzon *et al.*, 1997). The intense first exposure comprises a triple stress procedure including restraint-, forced submersion, and biological stress (exposure to ether or halothane vapours). This is followed by a brief re-stress procedure 7 days later (Harvey *et al.*, 2003a) that consists of a contextual reminder of the prior stress, using either swimming or water emersion stress (Uys *et al.*, 2003). The TDS model of PTSD aims to induce biological and behavioural changes, analogous to the major key symptoms of PTSD in a dose-dependent fashion (Pynoos *et al.*, 1996). In recent years, there has been significant progress in validating this particular animal model of repeated trauma with respect to the principles of face-, construct- and predictive validity (Harvey *et al.*, 2003b; Harvey *et al.*, 2004a; Harvey *et al.*, 2004b; Harvey *et al.*, 2005b).

2.7.1.3 *Inescapable shock-learned helplessness (IS-LH)*

Inescapable foot shock is a behavioural paradigm in which rats are exposed to a series of shocks from which the animal cannot escape, followed by their introduction to a paradigm from which they are able to escape (Yehuda & Antelman, 1993). Interestingly, it appears that animals that previously have been exposed to the inescapable shock lose their ability to escape even when this option is presented (Yehuda & Antelman, 1993). Such behavioural response to uncontrollable stress is referred to as "learned helplessness" (Maier, 2001), and describes

the “despair” that ensues following the realization that attempts to escape the stressor is pointless. This paradigm has been accepted as an animal model of PTSD (Maier, 2001; Overmier, 2002) even though it was initially designed for depression (Sherman & Petty, 1982).

2.7.2 Assessing animal models of PTSD: Paradigms for behavioural assessment

An essential additional element of any animal stress model is the assessment tools applied to measure the different aspects of behaviour emulated by the model (Ohl, 2003). Assessment tools that are often used in PTSD studies are those that quantify and characterize the constructs of memory, anxiety and learning. Intricate behavioural patterns, consisting of independent as well as interactive components such as motor, emotional as well as cognitive functions, are found in rats when responding to stress or aversive stimuli (Lesch *et al.*, 2003).

2.7.2.1 Pavlovian conditioning

A large number of animal models have been developed to imitate the traumatic events that cause the extreme symptoms and persistent fear that is a distinctive characteristic of PTSD. Briefly, in Pavlovian fear conditioning, rodents are trained to relate shock delivery with either a cue or a context, where memory for such association is later assessed by measuring freezing behaviour upon re-exposure to the conditioned cue in the absence of the shock (Cardinal *et al.*, 2002). Assessments related to fear conditioning in models of PTSD have a number of advantages since it may reflect the common clinical observations in PTSD patients. Re-experiencing of fear causes sensitization of fear over a period of time that ultimately results in maladaptive behavioural responses (Charney, 2004). Also, the amygdala, hippocampus and prefrontal cortex that have been implicated in the psychopathology of PTSD, are involved in processes of fear conditioning. Therefore, studies of fear conditioning have the potential to elucidate essential genes, cells as well as circuits that contribute to both normal and pathological fear (Bremner *et al.*, 2002). Another behavioural assessment that follows the same approach, albeit applying slightly different cues and aversive stimuli, is the conditioned taste aversion test (CTA), where animals learn to associate a novel taste (conditioned stimulus) with an aversive experience (Miranda *et al.*, 2003).

2.7.2.2 Elevated plus maze (EPM)

Behavioural avoidance and anxiety as is evident in PTSD can be modelled in rodents using the EPM (Carobrez & Bertoglio, 2005; see Figure 2-18). This behavioural test will be the primary screening test of anxiety applied in the current study. The procedure is based on the conflict between the inherent tendency of the rodent to explore and its aversion of open spaces. Briefly, the apparatus consists of a plus-shaped maze with two open arms (1m in length) and two closed arms (also 1m in length) raised 60 cm off the floor (Harvey *et al.*, 2006). Anxiety related behaviours, including the number of entries into open (OAE) and closed (CAE) arms, time spent in the open arms (sOA) and time spent in the closed arms (sCA), and head dipping behaviour are scored. It has been demonstrated that both forced and voluntary passage onto the open arms of the EPM is related to elevated plasma corticosterone concentrations, increased freezing, as well as higher numbers of faecal boli (Pellow *et al.*, 1985), changes that are indicative of increased anxiety. Normal exploratory behaviour is in favour of the closed arms; in fact, the tendency to remain within the closed arms can be reinforced by anxiogenic compounds (Handley & Mithani, 1984). In contrast, administration of anxiolytic compounds reduces the natural avoidance of the open arms and promotes the exploration there of.



Figure 2-18 - Visual representation of the elevated plus maze (<https://mazeengineers.com>)

2.7.2.3 Open field test (OFT)

The OFT test is widely used to assess emotionality (by defecation) and anxiety as well as to determine drug-related effects on locomotor activity in a rodent (Lister, 1990; see Figure 2-19). Briefly, the apparatus used for the OFT consists of a 1 m x 1 m x 0.5 m square test arena. The rat is placed in one corner of the arena and allowed to explore the environment for 11 minutes. The number of entries into the central square of the arena provides a measurement of non-anxiety-like behaviour (Möller *et al.*, 2011). Other assessments based on exploratory behaviour include the exploration box (Lister, 1990), hole poking board (Rodgers *et al.*, 1997), and the elevated T-maze (Rodgers *et al.*, 1997). Similarly, to the EPM, these tests assess the natural tendency of rodents to explore new environments and as well as fear of open, elevated, or unfamiliar spaces. In all of these tests, freezing or immobility is usually accepted as a sign of fear (Landgraf & Wigger, 2002). The OFT is also used to assess the effects of drugs on different aspects of the behaviour and its underlying motivation (Choleris *et al.*, 2001). Therefore, the number of times an individual undergoes testing in the OFT can significantly influence its behaviour as well as the underlying motivational construct. Upon numerous testing, animals quickly habituate to the open field, while their locomotion tends to decrease over different testing sessions until the activity reaches a stable level (Choleris *et al.*, 2001). This stabilized level of activity is often used to assess the effects of drugs on locomotion (Choleris *et al.*, 2001). Similarly, in other tests like the EPM, rodents also present with behavioural modifications as well as differential responses to treatment with anxiolytic agents upon re-testing (File *et al.*, 1993). This suggests that the motivational factors underlying animal behaviour upon first exposure to the OFT are very different from those that modulate behaviour during subsequent exposures. Thus, it can be hypothesized that, as in the EPM, the first exposure to the OFT elicits mainly anxiety-mediated behavioural responses, whereas subsequent exposures do not (Rodgers *et al.*, 1997). First exposure to the OFT can hence be used to measure the effects of various pharmacological agents on levels of anxiety (Choleris *et al.*, 2001).

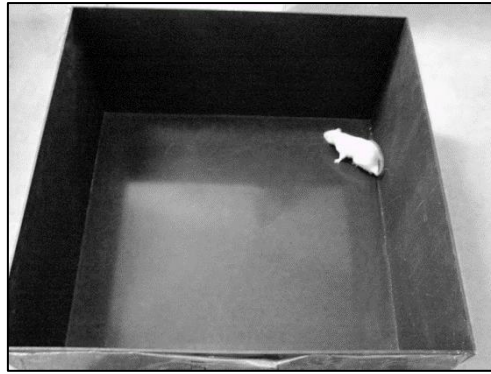


Figure 2-19 - Visual representation of the open field test (own photograph)

2.8 Conclusion to Chapter 2

As is true for most psychiatric conditions, PTSD remains an enigma while its diagnosis is challenged by many difficulties, including the high prevalence of comorbid disorders as well as the variation in PTSD symptomology (American Psychiatric Association, 2013). PTSD presents with symptoms of intrusive traumatic memories, re-experiencing of the trauma in the form of nightmares and flashbacks, persistent hyperarousal and inappropriate fearful responses to stimuli that are not, or are no longer, threatening (American Psychiatric Association, 2013). These memory interruptions can be upsetting and can accelerate the progression of acute stress disorder to PTSD. Re-experiencing of symptoms can cause reconsolidation of the traumatic memories that result in patients experiencing more vivid flashbacks and reliving the trauma. The invasive and vivid recollection of the early traumatic event can interfere significantly with the patient's overall ability to cope and is extremely detrimental to the social functioning of patients (Yehuda 2002), their physical health and emotional wellbeing (Woods, Wineman 2004). PTSD is aptly considered to be a disorder of memory and a syndrome of maladaptive fear responses to a traumatic stressor (Cohen *et al.*, 2003; Olf *et al.*, 2009).

Due to complications in treatment and treatment resistance (Horn *et al.*, 2016), it is of great importance to investigate novel strategies for the prevention and treatment of PTSD. The role of the noradrenergic system in conditioned fear responding represents an important biological target in the prevention and treatment of PTSD. In this regard, the greater potency of NA at the α_{2C} AR vs. the α_{2A} ARs and the differences in affinity of NA for these two receptors (Hein *et al.* 1999), is intriguing. Moreover, that selective inhibition of the α_{2C} AR has recently been found to offer significant cognitive benefits in rodent models of depression

and psychosis (Uys *et al.*, 2017), raises the question whether selective α_{2C} AR antagonists may offer clinical benefits in animal models characterised by hyperadrenergic states, such as PTSD (Yamamoto, Hornykiewicz 2004). Since the α_{2C} AR demonstrates lower expression in the medial PFC (Sallinen *et al.*, 2013) but a higher distribution in the striatum and hippocampus compared to the α_{2A} AR (Fagerholm *et al.*, 2004), ORM-10921, a selective and potent α_{2C} receptor antagonist, could be useful in exploring α_{2C} receptor physiology in rodents with PTSD.

In conclusion hyperadrenergic, hyperdopaminergic, hyperglutamatergic and hyposerotonergic activity is implicated in the neuropathology of PTSD. Further, reduced baseline cortisol levels and a hyper-responsive HPA negative feedback response is 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. The aim of this study will be to consider the association between selective antagonism of the α_{2C} AR and subsequent behavioural and neuroendocrine effects, as assessed in the PSEM of PTSD.

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3 MANUSCRIPT A

Article to be submitted to *European Neuropsychopharmacology* titled:

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Author Contributions

- *Crystal Erichsen* designed the investigation in consultation with Brian H Harvey and De Wet Wolmarans, performed all behavioural, pharmacological, and statistical analyses, wrote the first version of the manuscript, and edited the manuscript following input from the co-authors.
- *Dan J Stein* acted as clinical consultant with respect to experimental design and manuscript review.
- Brian H Harvey and De Wet Wolmarans were study supervisor and co-supervisor respectively, assisted in the interpretation of results. Brian H Harvey will also act as corresponding author in the submission of the final manuscript to *European Neuropsychopharmacology*.

Important Information

- Instructions to the author are included in Addendum A.
- All co-authors provided consent for the paper to be assessed as part of the M.Sc. dissertation of *Crystal Erichsen* (Addendum B).

The α_{2c} selective antagonist, ORM-10921, displays anxiolytic effects in an animal model of posttraumatic stress disorder

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Keywords: Animal model; posttraumatic stress disorder; α_2c ; corticosterone; anxiety; noradrenergic system

Abstract

Posttraumatic stress disorder (PTSD) is a severe trauma and stress related disorder that follows exposure to a life-threatening event, being diagnosed if the patient exhibits re-experiencing and avoidance phenomena, negative cognitions and mood, and arousal. Supported by clinical and basic neuroscience research, the neurobiology of PTSD is linked to abnormal activity of the hypothalamic-pituitary-adrenal (HPA) axis as well as noradrenergic hyperfunction. Current treatments for PTSD are inadequate, while animal models have become critical to study the mechanisms underlying trauma-induced changes, the physiological and neurobiological aetiologies underlying PTSD, as well as being a platform to test novel drug treatments. Modulating the noradrenergic system has attracted substantial interest in the treatment of PTSD, while recent studies have emphasised that non-selective modulation of the α_2 adrenoceptor (AR) may exert opposing actions via the α_{2A} and α_{2C} AR. The aim of the study was to validate the predator scent exposure (PSE) model of PTSD with respect to behavioural changes comparable to that in human PTSD, and to explore the putative therapeutic effects of ORM-10921, a selective α_{2C} AR antagonist, in the model. Male Wistar rats were subjected to PSE for 10 min, and subsequently treated chronically with either saline or ORM-10921 (0.3 mg/kg/day) administered subcutaneously for 21 days. PSE induced significant anxiety-related behaviour, as assessed during PSE, as well as in the elevated plus maze (EPM) on days 7 and 21 post-PSE, while inducing a trend towards reduced plasma corticosterone levels on day 21. ORM-10921 reduced PSE-induced anxiety without markedly affecting plasma corticosterone compared to saline treated PSE rats. This study has demonstrated that ORM-10921 presents with therapeutic utility in treating PTSD when used as an immediate post-trauma intervention, emphasizing the importance of the α_{2C} AR in PTSD and its treatment.

I Introduction

PTSD is an anxiety disorder induced by exposure to a traumatic and life-threatening event (Rauch and Foa, 2003). As such, PTSD has recently been classified by the American Psychiatric Association (APA) as a trauma and stress related disorder (American Psychiatric Association, 2013) as opposed to its previous status as an anxiety disorder (American Psychiatric Association, 2013). The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) pays attention to the behavioural symptoms that accompany PTSD and proposes four instead of three distinct diagnostic clusters. These are described as re-experiencing, avoidance, negative cognitions and mood, and arousal (American Psychiatric Association, 2013).

Current pharmacological treatments for PTSD include antidepressants, i.e. selective serotonin reuptake inhibitors (SSRIs) (Vaswani *et al.*, 2003), serotonin and norepinephrine reuptake inhibitors (SNRIs) (Vaswani *et al.*, 2003), tricyclic antidepressants (TCAs) (Asnis *et al.*, 2004) and monoamine oxidase inhibitors (MAOIs) (Borsini *et al.*, 2002). However, 40% of patients with PTSD do not meet the typical response criteria following an initial course of treatment (Stein *et al.*, 2006), while the majority of patients present with residual symptoms after monotherapeutic intervention (Stein *et al.*, 2006). Moreover, the remission rate following treatment with sertraline (an SSRI and the only FDA approved antidepressant to treat PTSD), has been reported at 25% (Davidson *et al.*, 2004). As such, many patients remain refractive to treatment (Albucher & Liberzon, 2002; Schoenfeld *et al.*, 2004; Ravindran & Stein, 2009). A better understanding of the neurobiology of PTSD may support the development of improved and effective treatments for PTSD (Hamner *et al.*, 2004).

Of particular note, not all individuals exposed to trauma will progress to full-blown PTSD, with only 20-40% of victims subjected to the same trauma developing the illness (Breslau *et al.*, 1991; Shalev, 2000; Cohen *et al.*, 2004). This is also supported by preclinical investigations, where only 15–25% of trauma-exposed rodents present with PTSD-like manifestations (Cohen *et al.*, 2004). These findings therefore propose a role for differences in individual resilience profiles that contribute to the risk of developing PTSD (Cohen *et al.*, 2004). From a neurobiological perspective, preclinical and clinical studies have provided strong evidence that neuropeptide Y (Morgan *et al.*, 2002), and the monoamines serotonin (5HT) (Krystal & Neumeister, 2009) and noradrenalin (NA) (Krystal & Neumeister, 2009), are implicated in PTSD risk, while the hypothalamic-pituitary-adrenal (HPA) axis hormone, cortisol, has also

notable for its role in PTSD risk vs. resilience (Yehuda *et al.*, 2007). Other factors include gender, age at trauma, and race that predict PTSD in some populations but not in others, while factors such as education, previous trauma, and general childhood adversity predict PTSD more constantly. Individually, the effect size of all the risk factors was modest, but factors operating during or after the trauma, such as trauma severity, lack of social support, and additional life stress, present with somewhat stronger effects than pre-trauma factors (Brewin *et al.*, 2000).

Since PTSD is a disorder of memory, involving increased consolidation of trauma-related memory, compromised extinction of such fear memory and the genesis of fear and anxiety-related manifestations, the inter-relationship between the noradrenergic system and the HPA-corticosterone axis in mediating these effects is a worthy biological target for investigation (Kanter *et al.*, 2001). Noradrenergic activation in the basal lateral amygdala (BLA) plays a central role in the physical expression of fear and anxiety (Quirarte *et al.*, 1997; Roozendaal *et al.*, 1999), while blocking β ARs in the BLA prevents glucocorticoid-induced avoidance behaviour and fear memory (Quirarte *et al.*, 1997; Roozendaal *et al.*, 2002). Indeed, norepinephrine infused into the BLA immediately post trauma enhances memory through activation of β - and α_1 -adrenoceptors (AR) (Liang *et al.*, 1986; Liang *et al.*, 1990; Ferry & McGaugh, 1999; Ferry *et al.*, 1999a; Roozendaal *et al.*, 2002). These findings concur that fear-related anxiety and other manifestations are mediated by noradrenergic activation in the amygdala and the subsequent dysregulation of the HPA-axis, leading to disordered cortisol release (hyper- and hypocortisolemia) and subsequent adverse effects.

Based on its intricate association with the biological stress response, the noradrenergic-locus coeruleus (LC) system (Charney, 2004) and noradrenergic manipulation has been the subject of numerous investigations in PTSD (Morgan *et al.*, 2002; Krystal & Neumeister, 2009; Hendrickson & Raskind, 2016). Considering the prominence of hyperadrenergic symptoms in PTSD (e.g. hyperarousal, re-experiencing, anxiety, tachycardia, increased diastolic blood pressure, diaphoresis; (Krystal & Neumeister, 2009), there is now considerable evidence supporting abnormal regulation of central NA signalling in PTSD, both in humans (Southwick *et al.*, 1993; Bremner *et al.*, 1997; Southwick *et al.*, 1997; O'Donnell *et al.*, 2004a; O'Donnell *et al.*, 2004b; Shin *et al.*, 2006) and animals (Arnsten *et al.*, 1999; Harvey *et al.*, 2006). In this regard, the $\beta_{1/2}$ AR blocker, propranolol and the α_1 AR blocker, prazosin, are effective for treating certain PTSD-related manifestations, e.g. amplified startle responses, nightmares and

intrusive re-experiencing (Friedman, 1997; Taylor & Raskind, 2002). Furthermore, studies have demonstrated the effect of agonists and antagonists of the α_2 AR in PTSD (Liang *et al.*, 1986; Liang *et al.*, 1990; Ferry & McGaugh, 1999; Ferry *et al.*, 1999b; Ferry *et al.*, 1999a; Krystal & Neumeister, 2009) or in related models in animals (McIntyre *et al.*, 2002), showing that reduced noradrenergic activity in the amygdala inhibits memory consolidation, and β - and α AR agonism in the BLA to enhance memory consolidation (Liang *et al.*, 1986; Liang *et al.*, 1990; Ferry & McGaugh, 1999; Ferry *et al.*, 1999b; Ferry *et al.*, 1999a). Previously, the $\beta_{1/2}$ AR antagonist, propranolol, was found to lower increased startle response, nightmares and intrusive re-experiencing in patients with PTSD (Vaiva *et al.*, 2003), while the α_1 -adrenoceptor antagonist prazosin, may effectively treat sleep disturbances in patients with chronic PTSD (Taylor & Raskind, 2002). Both drugs inhibit excessive noradrenergic activity that is associated with fear and startle responses that in turn result in heightened emotional arousal in PTSD. The non-selective α_2 AR agonists, clonidine and guanfacine, have been found to reduce nightmares, insomnia, intrusive recollections, startle reactions and hypervigilance in patients with PTSD (Harmon & Riggs, 1996; Horrigan & Barnhill, 1996). However, non-specific targeting of α ARs may result in unpredictable treatment response, often with opposite results (Sallinen *et al.*, 1999; Uys *et al.*, 2017b). Nevertheless, investigating the therapeutic potential of selectively targeting certain α_2 AR subtypes has distinct merit (Uys *et al.*, 2017a). Indeed, transgenic mouse experiments suggest that the α_{2C} AR has emerged as a target of specific interest in neuropsychiatry, with recent work focusing especially on mood, psychosis and Alzheimer's disease (Uys *et al.*, 2017a). With anxiety recognised as being a co-morbid diagnosis in all three the above noted disorders (Garner *et al.*, 2009; Rinne *et al.*, 2017), and given the prominent role of NA in anxiety and PTSD noted above, selectively targeting the α_{2C} AR may represent an important novel approach to treating PTSD (see Figure 1).

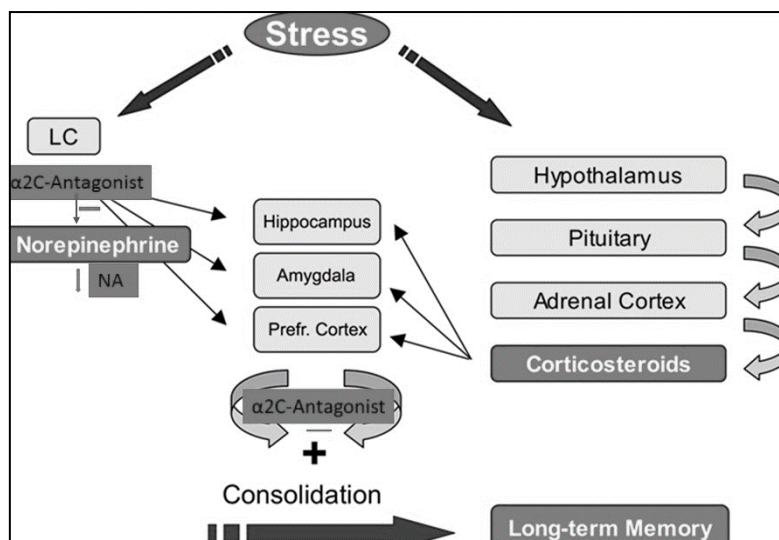


Figure 1 – Exposure to a fearful event promptly activates the autonomic nervous system and enhances norepinephrine levels in the brain. At the same time the hypothalamic–pituitary–adrenal (HPA) axis is activated which causes a slow increase in plasma corticosterone levels. These hormones act via receptors in regions that are critical for memory formation, i.e. hippocampus, amygdala, and prefrontal cortex, where they promote memory consolidation. Blocking the effects of noradrenalin via α_{2C} AR antagonist may interfere with memory by disrupting consolidation of the short-term memory to long-term memory. (Adapted from Uys et al 2017)

Activation of both α_{2A} and α_{2C} AR inhibit the release of NA (Hein et al. 1999), although the α_{2C} -receptor system elicits a weaker inhibitory response compared to α_{2A} AR (Bücheler et al. 2002). Important however, is that the potency of NA at the α_{2C} AR is higher than that at the α_{2A} AR, correlating with the difference in affinity of NA for the two receptors (Hein et al. 1999). Moreover, opposing actions of α_{2C} AR vs. α_{2A} AR antagonists with respect to mood and cognitive parameters have been reported, indicating that non-selective α_2 AR antagonism may be counter-productive (see Uys et al, 2017a for review). Further, animal studies have demonstrated that over expression of α_{2C} receptor (α_{2C} -OE) bolsters depressive-like behaviour and increases corticosterone levels following repeated stress, while knock out (KO) or absence of α_{2C} AR (α_{2C} -KO) exerts the opposite effects (Sallinen et al., 2007). Further, α_{2C} -KO mice demonstrate protective effects on stress while α_{2C} -OE mice are stress-sensitive (Sallinen et al., 2007). These findings are supported by neurobiological data demonstrating decreased serotonin (5HT), 5-hydroxyindoleacetic acid (5HIAA), 3-4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in α_{2C} -KO, compared to α_{2C} -OE subjects (Sallinen et al., 1999), and are congruent with data from clinical investigations regarding associations between the stress response and monoaminergic alterations (Southwick et al., 2007; Krystal & Neumeister, 2009). Therefore, subtype selective α_{2C} AR antagonists may play a unique and important role in the manipulation of post-trauma anxiety and other cognitive parameters.

The predator scent exposure (PSE) model (Cohen *et al.*, 2000; Cohen *et al.*, 2006) resembles a natural paradigm for inducing psychogenic stress (Adamec *et al.*, 1997). It has ethological relevance as it comprises an intense traumatic experience and results in long term changes in behavioural, autonomic and hormonal responses in rats that correlate with the symptoms of PTSD in humans (Cohen *et al.*, 2003). The model presents with good face validity for PTSD in that it reproduces key behavioural manifestations of the disorder, i.e. inter-animal variance in symptomological presentation (Cohen *et al.*, 2003), hyperarousal (Hebb *et al.*, 2003; Cohen *et al.*, 2004; Cohen *et al.*, 2006; Cohen *et al.*, 2008; Lewitus *et al.*, 2008), increased levels of anxiety (Adamec *et al.*, 2006; Cohen *et al.*, 2008; Muñoz-Abellán *et al.*, 2008), social withdrawal (Zangrossi & File, 1992) and increased freezing and avoidance behaviour (Blanchard *et al.*, 1990a; Zangrossi & File, 1992; Wallace & Rosen, 2000; Dielenberg *et al.*, 2001; Dielenberg & McGregor, 2001; Masini *et al.*, 2005). Furthermore, the model presents with similarities with regard to the molecular and biological bases of PTSD, including altered glutamatergic and noradrenergic signalling, increased sympathetic activity and increased adrenocorticotrophic hormone (ACTH) concentrations, diminished vagal tone and a shift in the sympathovagal balance (Cohen *et al.*, 2003), enhanced sensitivity to negative glucocorticoid feedback, and altered corticosterone levels (Cohen *et al.*, 2003). Finally, propranolol (Ferry *et al.*, 1999a; Pitman *et al.*, 2002; Vaiva *et al.*, 2003), steroid synthesis inhibitors, e.g. ketoconazole (Cohen *et al.*, 2000), chronic antidepressant treatment, as well as alcohol (Blanchard *et al.*, 1990b), attenuate stress-related behaviours in this model, as has been demonstrated in clinical PTSD (Blanchard *et al.*, 1990b).

Various clinical (Strawn & Geraciotti, 2008) and preclinical (Bryant *et al.*, 2009; Holbrook *et al.*, 2010) studies have revealed the potential of targeting noradrenergic receptors, especially the immediate post-trauma use of the $\beta_{1/2}$ AR antagonist, propranolol, to prevent the neurodevelopment of PTSD over time (Vaiva *et al.*, 2003). However, the effects of targeting the α_{2c} AR in PTSD remains unexplored, mainly since ligands for the α_{2c} AR subtype have until recently been unavailable (Uys *et al.*, 2017a). The aim of this study was to explore whether the typical anxiety-related manifestations engendered by PSE are reversed following sub-chronic treatment with the selective α_{2c} receptor antagonist, ORM-10921. As such, the present investigation will : 1) establish and characterize the bio-behavioural responses of PSE (that of a male domestic cat) in male Wistar rats through the presentation of anxiety-like manifestations akin to that in clinical PTSD, 2) compare the effects of immediate post-exposure intervention with chronic ORM-10921 treatment vs. a vehicle control to reverse

anxiety-like manifestations on days 7 and 21 post-trauma, and 3) determine endpoint differences in plasma corticosterone concentrations elicited by 21-day treatment with either a vehicle control or ORM-10921 in both the non-PSE exposed and PSE exposed groups.

2 Experimental procedure

2.1 Animals

Wistar rats demonstrate a more robust response to conditioned and unconditioned stress, compared to Sprague-Dawley rats (Staples & McGregor, 2006; Uys *et al.*, 2016). As such, 122 male Wistar rats weighing 150 – 200g were used in the current investigation. They were housed in individually ventilated (230 [h] x 380 [w] x 380 [l] mm) cages in groups of 4 – 5 per cage and handled every day for 5 minutes for 10 days prior to experimental analyses. Bedding material was provided in the form of corncob and cages were maintained on a 12h light/dark cycle at 21°C and relative humidity of 50 ± 10%. Food and water were provided *ad lib*. Animals were bred, supplied, and housed at the Vivarium (SAVC reg. no. FR15/13458; SANAS GLP compliance no. G0019) of the Pre-Clinical Drug Development Platform of the North-West University. All experiments were approved by the AnimCare Animal Research Ethics Committee (NHREC reg. no. AREC-130913-015) of the North-West University (Ethics approval number NWU-00438-16-S5).

2.2 Validation and Application of Predator Scent Exposure as a Trauma-related Interference

2.2.1 Behavioural validation of PSE as a potential model of PTSD in our laboratory

Given that approximately 25-40% of individuals (human or animal) exposed to trauma will progress to full-blown PTSD (Breslau *et al.*, 1991; Cohen *et al.*, 2003), we aimed to confirm such a response in trauma-exposed animals by determining the percentage of animals showing afflicted vs. non-afflicted behaviour as measured in the elevated plus maze (EPM) (Adamec *et al.*, 1998) on day 7 post-PSE. In this regard, we identified the extreme ends of normal distribution of anxious behaviour in response to PSE to validate the PSE model, as described below. That said, the faecal boli counted immediately after 10-minute to either cat scented or non-cat scented cloth and was used as a measure of immediate trauma-induced anxiety.

Well-adapted Cohort

Based on column analyses of data generated in the EPM, well-adapted animals were defined as those individuals clustered within the upper quartile of the normal distribution relating to the number of entries into and time spent in the open arms (i.e. the extreme of less anxious behaviour), and/or being clustered within the lower quartile of the normal distribution relating to the number of entries into and time spent in the closed arms (i.e. the extreme of less anxious behaviour).

Maladapted Cohort

Based on column analyses of data generated in the EPM, maladapted animals are defined as those individuals clustered within the lower quartile of the normal distribution relating to the number of entries into and time spent in the open arms (i.e. the extreme of high anxiety behaviour), and/or being clustered within the upper quartile of the normal distribution relating to the number of entries into and time spent in the closed arms (i.e. the extreme of high anxiety behaviour).

2.2.2 *Determining the response of predator scent non-exposed and exposed behaviours to immediate, but chronic drug interference*

As opposed to the methods followed in paragraph 2.2.1, no separation between maladaptive and well-adaptive cohorts was done as rats were subjected to injection stress (mode of administration of treatments) beginning 1 hour after PSE. However, to establish the outcomes of treatment, group behavioural comparisons were performed on day 7 and day 21.

2.3 *Animal Groups*

Rats were randomly assigned to 6 groups, as described in Figure 2:

2.3.1 *Groups used for validating PSE as a potential model of PTSD*

- Group I was exposed to clean unscented cloth only on day 0, and assessed on day 7 (n = 31) to validate the predator scent exposure model in our laboratory;
- Group II was exposed to cat scented cloth only on day 0, and assessed on day 7 (n = 31); to validate the predator scent exposure model in our laboratory.

2.3.2 Groups used to determine the response of predator scent non-exposed and exposed behaviours to immediate, but chronic drug interference

- Group III received vehicle drug (for 21 days) 1 hour after first exposure to clean unscented cloth on day 1, and assessed on day 7 and 21 (n = 8);
- Group IV received vehicle drug (for 21 days) 1 hour after first exposure to cat scented cloth on day 1, and assessed on day 7 and 21 (n = 12);
- Group V received ORM (0.3 mg/kg for 21 days) 1 hour after first exposure to clean unscented cloth on day 0, and assessed on day 7 and 21 (n = 16); and
- Group VI received ORM (0.3 mg/kg for 21 days) 1 hour after first exposure to cat scented cloth on day 0, and assessed on day 7 and 21 (n = 24).

2.4 Exposure

The exposure and treatment lay-out is presented in Figure 3. On day 0, predator scent exposures were carried out individually under dim white light (15 lux) for 10 min. Exposures involved subjecting animals to a 10 cm x 10 cm clean unscented cloth (Groups I, III and V; *hereafter referred to as the non-exposed cohort*) or cat scented cloth that had been exposed to a male domestic cat for 2 months (Groups II, IV and VI; *hereafter referred to as the exposed cohort*; Cohen *et al* 2003; Cohen *et al.* 2005, Yehuda 2009). Cloths were introduced in the outer corners of exact replicas of the normal housing cages where after rats were allowed to freely explore the cage for a period of 10 min. At the end of the exposure period, rats were returned to their home cages. Exposure cages were cleaned between trials and new cloths (10 cm x 10 cm) added for each exposure session.

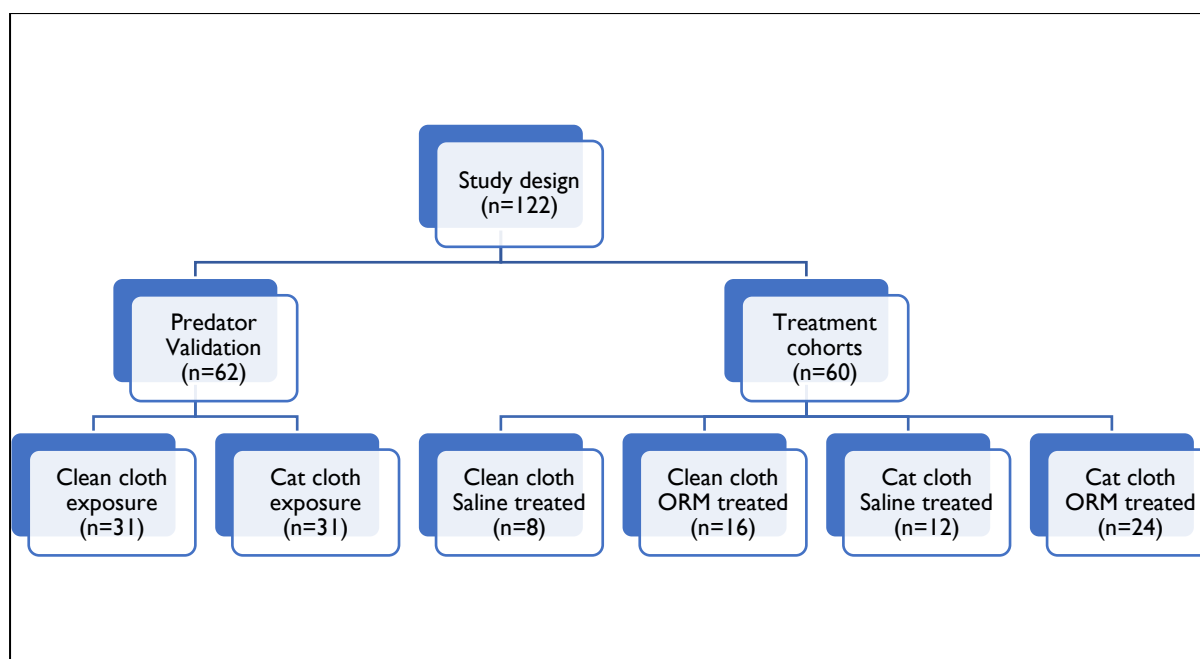


Figure 2 – Study design indicating the allocation of treatment and exposure groups

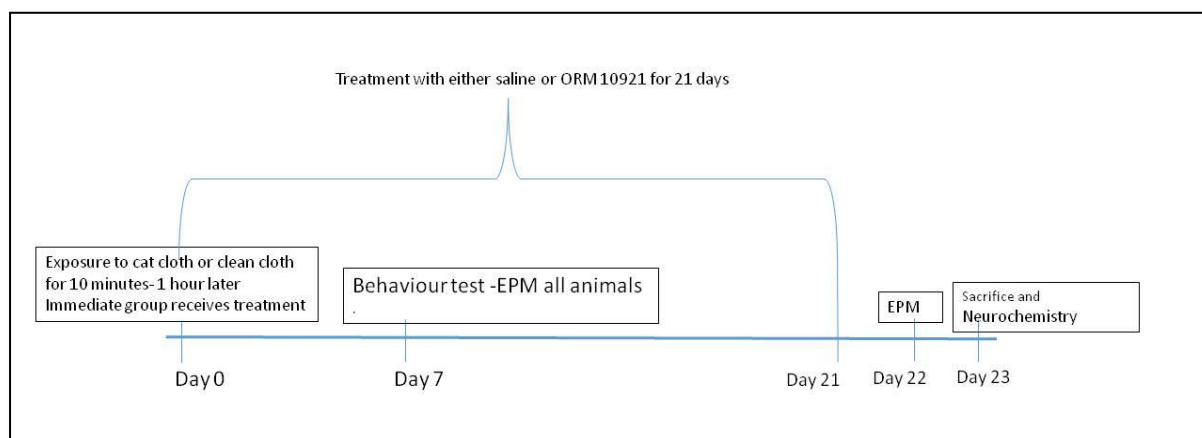


Figure 3 – Time line of study, indicating day 0 as day of exposure where the treatment cohorts were treated 1 hour after treatment for 21 days, all animals were evaluated in the elevated plus maze on days 7 and 21 and sacrificed on day 23. Outline shows PSE exposure, time of drug administration and duration of treatment, and time point of behavioural and neuroendocrine analysis.

2.5 Drugs and Reagents

ORM-10921 was a kind sponsorship from Orion Pharma, Turku, Finland. Assay kits for the analysis of plasma corticosterone were obtained from Abcam, Johannesburg, South Africa.

Vehicle (normal saline) or drug treatment were administered for 21 days (days 0 – 21), commencing 1-hour post PSE. Both vehicle and ORM-10921 were administered in a single subcutaneous dose of 1 ml/kg (or 0.3 mg/kg). Uys and colleagues(2017b) demonstrated

0.3 mg/kg ORM-10921 to have noteworthy antidepressant and pro-cognitive actions in a genetic animal model of depression (Uys *et al.*, 2017b) both symptoms of which are manifest in patients with PTSD (Association, 2013).

2.6 Assessment of Anxiety in the Elevated Plus Maze (EPM)

Symptoms of behavioural avoidance and anxiety can be assessed in rodents using the EPM (Carobrez & Bertoglio, 2005), which is based on the conflict between the rat's inherent need to explore novel surroundings and anxiety-like aversion of open spaces. The assessment of anxiety was conducted as described previously (Pellow *et al.*, 1985; Harvey *et al.*, 2006), with slight modification. The apparatus consists of a plus-shaped maze with two open arms and two closed arms perpendicular to each other, i.e. both open arms forms one line in the plus shape, while the closed arms form the other. All experiments in the EPM were carried out under dim light conditions (10 lux) while video tracking was facilitated via infrared backlight.

Performance in the EPM was video recorded for 5 min using digital cameras. Each animal used to validate PSE as a potential model of PTSD (Groups I and II) was subjected to testing in the EPM only on day 7 post PSE. Animals used to determine the outcomes of treatment (Groups III – VI) were assessed in the EPM twice, viz. day 7 and day 21 post-exposure. Anxiety-related behavioural parameters scored included number of entries into the open (OAE) and closed (CAE) arms, as well as the percentage time spent in the open (sOA) and closed (sCA). Head-dipping behaviour in the open arms was applied as a measure of risk assessment and exploratory behaviour. Head dipping occurs in the central area of the maze and reflects an aversion to risk taking, which is related to the anxious state of the animal (Walf & Frye, 2007). These parameters were scored and analysed using Ethovision XT13® (Noldus Information Technology, Wageningen, The Netherlands). Arm entries, as opposed to head-dipping episodes, were considered if all four paws of the animal entered a specific arm (Harvey *et al.*, 2006). Following each test, animals were returned to their home cages and the apparatus cleaned with 10% ethanol to eliminate odour trails.

2.7 Blood Collection and Neuroendocrine Analyses

On the morning following the last behavioural assessment in the EPM (*viz.* day 23), animals were decapitated, and trunk blood collected in pre-chilled, 4 ml vacutainer tubes (SGVac) containing K₂EDTA as anti-coagulant. Samples were centrifuged at 5000 rpm at 4°C for 10 min, and the plasma stored at -80 °C until the day of corticosterone analysis.

2.7.1 Corticosterone analysis

Corticosterone levels were determined in plasma using a Coat-A-Count Rat Corticosterone Elisa kit(TKRCI) supplied by Abcam. The ab108821 Corticosterone Elsia kits are designed for the quantitative measurement of corticosterone levels in plasma, serum, urine, milk, saliva and cell culture supernatant. On the day of analysis plasma samples and all materials and prepared reagents were equilibrated at room temperature. All reagents, working standards (6) and samples were prepared according to the manufacture's protocol. 25 µl of corticosterone standard was added to a well followed by 25 µl of biotinylated corticosterone protein to generate a stock solution. The plate was gently tapped to mix. Wells were covered and incubated for 2 hours at room temperature. After 2 hours of incubation, the wells were washed 5 times with 200 ul of wash buffer manually and tapped on an absorbent paper towel to remove liquid. 50 µl of SP conjugate was added to each well and gently tapped to coat the wells, followed by incubation for 30 minutes. The plates were then washed as described above, whereupon 50 µl chromogen substrate was added per well and incubated for 20 minutes.

After incubation, 50 µl of stop solution was added to each well. The colour changed from blue to yellow during the course of the reaction, with absorbance determined in a microplate reader and read at a wavelength of 450 nm, as described in the manufacturer's protocol. See (www.abcam.com/ps/products/108/ab108821/documents/products/ab108821%20Corticosterone%20Elisa%20kit%20v10%20) for more detail on kits

2.8 Statistics

All statistics were performed with IBM SPSS version 25 and GraphPad Prism® version 6 under the guidance of the statistical consultation service of the NWU. For the initial behavioural characterization of the non-exposed and exposed cohorts (Groups I and II), column statistics were applied to determine the upper and lower 25th percentiles of the distribution for each behavioural parameter measured in the EPM, while unpaired parametric (data distributed normally) or non-parametric (data abnormally distributed) student t-tests were used to

compare differences in the average behavioural manifestations between the non-exposed and exposed cohorts.

To compare the differences in treatment response with respect to EPM behaviours between the non-exposed and exposed cohorts on day 7 and 21 post-trauma respectively, mixed model analyses have been applied. Rats were treated as subjects of repeated measures while the covariance structure was kept unstructured. Also, as with the initial behavioural validation, column statistics were also applied to determine the pre- and post-treatment 25th quartiles of distribution. Due to the use of interquartile ranges as indicators of behavioural distribution, data in Figures 8 – 10 are represented with respect to the median and interquartile range, while being discussed considering both these parameters, as well as mean $\pm$ SEM. Mixed model analyses were followed with Cohen's d calculations as well as paired or unpaired Mann-Whitney U -tests (for non-parametric analysis) to determine the effect size and associated significance of demonstrable differences.

The correlation between anxiety, as assessed in the EPM on day 7 and 21, and plasma CORT on day 21 was examined using Spearman (if data shows no normality) and Pearson correlation (If data shows normality) analysis. The effect size is represented by Pearson's correlation coefficient (r), with $0.1 \leq r^2 < 0.3$ indicating a small effect, $0.3 \leq r^2 < 0.5$ a medium effect, and $r^2 \geq 0.5$ a large effect. A probability level of 95% was used to determine statistical significance ($p < 0.05$).

3 Results

3.1 Validation of the predator scent exposure model

During the PSE session, cat-cloth-exposed rats produced significantly more faecal boli compared to rats exposed to non-scented cloth immediately post-trauma, i.e. on day 0 (**Figure 4**: 4.8 ± 2.1 vs. 2.4 ± 1.4 , $p < 0.0001$). Furthermore, delayed manifestations of trauma were confirmed in at least a quarter (8/31) of PSE animals, but not the non-PSE groups, 7 days post exposure. As such, it was found that animals in the PSE cohort made significantly less entries into the open arms (**Figure 5**: 12.9 ± 5.1 vs. 8.7 ± 5.6 , $p = 0.0034$), and spent significantly more time in the closed arms (sCA) (**Figure 8**: 155.4 ± 14.5 vs. 115.7 ± 7.6 , $p = 0.01$), compared to animals of the non-PSE cohort. Although a comparison of the timespent in the open arms (sOA) (**Figure 7**: 85.6 ± 13.9 vs. 115.7 ± 10.2 , $p = 0.08$) failed to reach statistical significance, at least a quarter (8/31) of the exposed animals spent less time in the open arms (sOA) compared to any of the non-exposed animals (**Figure 7**: lower quartiles of

distribution: 14.6 s vs. 58.8 s). The same pattern was observed with respect to the number of open arm entries (**Figure 4**: lower quartiles of distribution: 4 vs. 10). Moreover, with respect to timespent in the closed arms (sCA), at least a quarter of exposed individuals remained in the closed arms of the EPM for longer than any of the individuals in the non-exposed cohort (**Figure 8**: upper quartiles of distribution: 229.8 s vs. 143.6 s). With respect to risk assessment behaviour, PSE animals engaged in significantly less risk-assessment behaviour compared to non-exposed individuals as indicated by a marked reduction in the number of head dipping episodes (**Figure 9**: 7.87 ± 1.26 vs. 16.94 ± 2.36 , $p = 0.0013$).

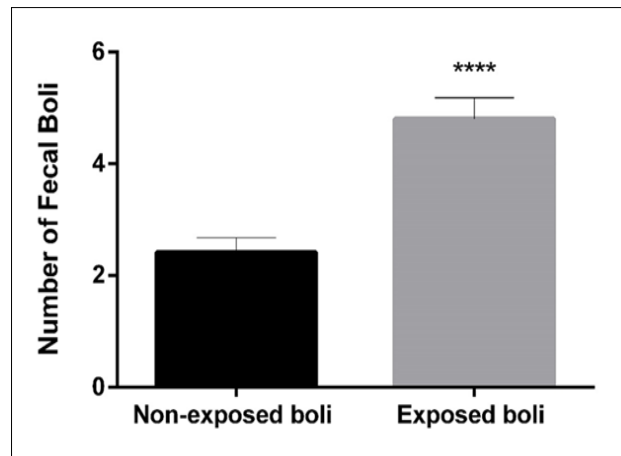


Figure 4 – Number of faecal boli produced by exposed vs. non-exposed individuals immediately after PSE (4.8 ± 2.1 vs. 2.4 ± 1.4 , $p < 0.0001$).

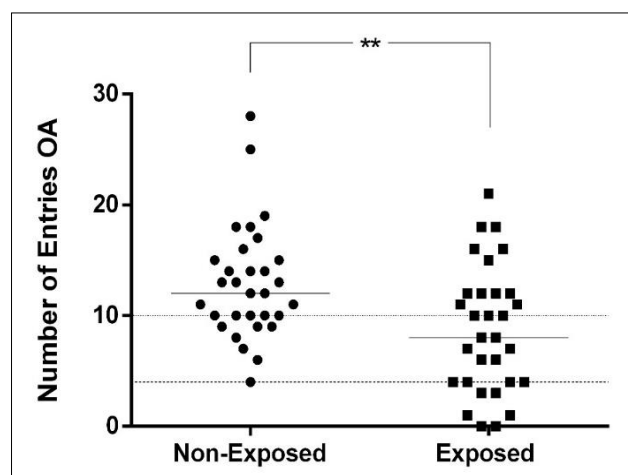


Figure 5 – Number of open arm entries made 7 days after PSE in exposed vs. non-exposed individuals; horizontal lines indicate the lower quartiles of distribution (12.9 ± 5.1 vs. 8.7 ± 5.7 , $p = 0.0034$)

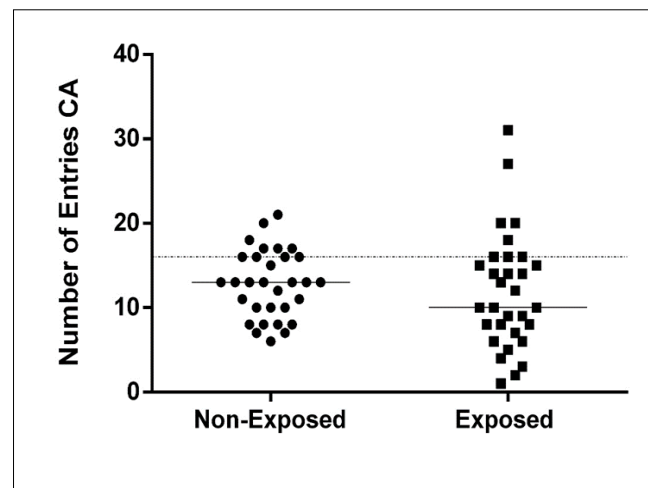


Figure 6 – Number of closed arm entries made 7 days after PSE in exposed vs. non-exposed individuals; horizontal lines indicate the upper quartiles of distribution

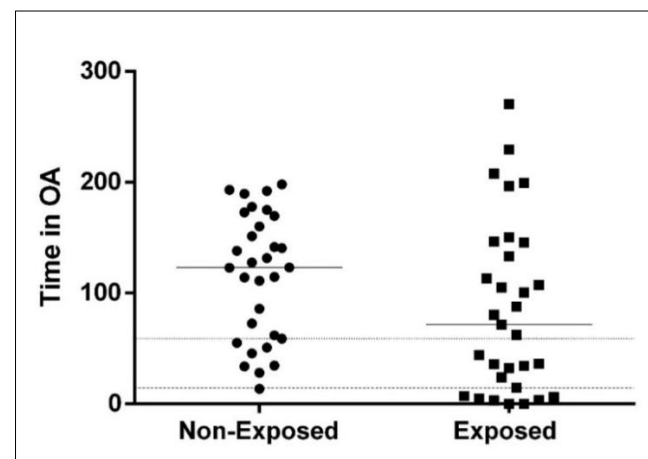


Figure 7 – Time spent in the open arms 7 days after PSE in exposed vs. non-exposed individuals; horizontal lines indicate the lower quartiles of distribution

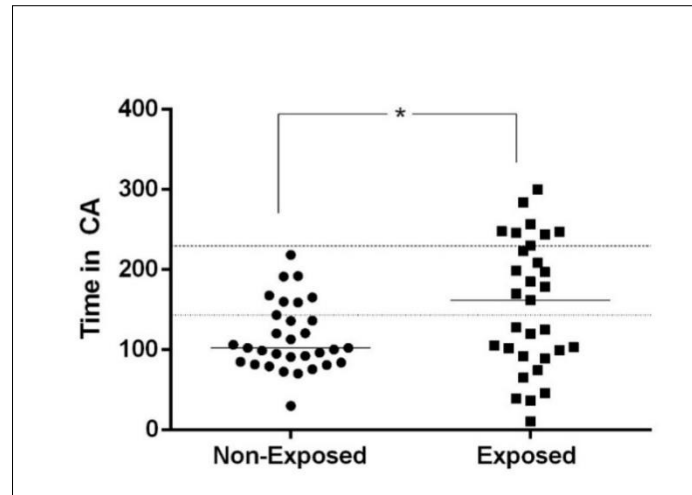


Figure 8 – Time spent in the closed arms 7 days after PSE in exposed vs. non-exposed individuals; horizontal lines indicate the upper quartiles of distribution

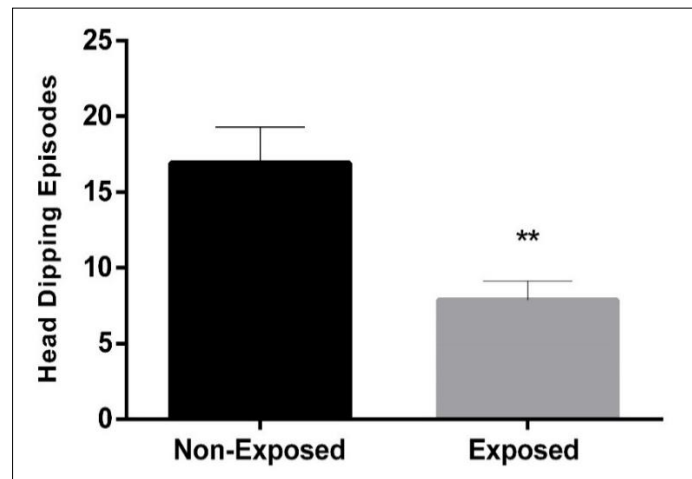


Figure 9 – Number of head dipping episodes expressed 7 days after PSE in exposed vs. non-exposed individuals

3.2 Behavioural responses of non-exposed and PSE individuals to ORM-10921

According to the data presented above PSE presents with anxiety like behaviour in the exposed but not in the non-exposed animals. This model was used to further determine pharmacological effects of ORM-10921 by assessing behaviour in the EPM on days 7 and 21. No separation of the afflicted was undertaken here, as noted earlier.

3.2.1 Open arm behaviour

3.2.1.1 Open arm entries (Figure 10A)

Following mixed model analysis to examine the effects of exposure (control; PSE), time (Day 7; Day 21), and treatment (Saline; ORM-10921) on the number of open arm entries (NOAE) in the EPM, no significant three-way interaction could be demonstrated [$F(1, 56) = 0.027$, $p = 0.869$]. However, a statistically significant simple two-way interaction was found between treatment and time [$F(1, 56) = 16.457$, $p = 0.0005$]. As such, in the saline treated control, the number of entries into the open arms changed significantly over the course of 21 days in both the non-exposed [4.4 ± 1.4 , $M(3.0)$, 75th(8.2) vs. 10.9 ± 0.9 , $M(10.0)$, 75th(13.5); $d = 1.62$] and exposed cohorts [6.1 ± 1.1 , $M(5.5)$, 75th(9.0) vs. 12.7 ± 1.2 , $M(11.5)$, 75th(16.7); $d = 1.64$], with the number of entries into the open arms decreasing with an effect size of 1.3-2.0 in exposed vs. non exposed animals (Figure 10A). However, time had no effect on the open arm behaviour of either the non-exposed [8.00 ± 0.89 , $M(7.00)$, 75th(10.7) vs. 9.4 ± 0.6 , $M(9.5)$, 75th(12.0); $d = 0.36$] or exposed [7.08 ± 0.89 , $M(7.50)$, 75th(10.7) vs. 9.0 ± 1.0 , $M(9.5)$, 75th(13.0); $d = 0.48$] cohorts treated with ORM-10921, although reduced entries into the open arms of large practical significance was evident in ORM-10921 treated rats on day 21 for non-exposed animals and day 7 for exposed animals, compared to their respective vehicle treated controls (Figure 10A; non-exposed: $d = 2.99$; exposed: $d = 3.6$). Detailed descriptions of interquartile ranges, Cohen's d calculations, and Mann-Whitney statistics, are provided in Tables 1 – 3.

3.2.1.2 Time spent in the open arms (Figure 10B)

A significant three-way interaction between exposure, time, and treatment could not be demonstrated for time spent in the open arms [$F(1, 56) = 0.025$, $p = 0.87$]. Further, simple two-way interactions were also not found between either treatment and time [$F(1, 56) = 0.253$, $p = 0.62$], exposure and time, [$F(1, 56) = 1.768$, $p = 0.19$] or exposure and treatment [$F(1, 56) = 1.38$, $p = 0.25$]. However, as evinced by a Cohen's d of 1.32, chronic treatment with saline significantly reduced the time spent in the open arms over the course

of time in the non-exposed cohort [38.5 ± 16.5 , M (11.80), 75th (89.60) vs. 112.2 ± 19.2 , M (120.7), 75th (164.8), $U = 9$, $p = 0.01$], with no such demonstrable effect observed in the exposed cohort [$d = 0.95$] (**Figure 10B**). The same trend was observed in the ORM-treated groups [non-exposed: 60.3 ± 11.4 , M (42.3), 75th (95.8) vs. 124.7 ± 17.0 , M (135.3), 75th (166.5); $d = 1.16$, $U = 55$, $p = 0.005$] and exposed cohort [55.96 ± 9.267 , M (51.9), 75th (81.50) vs. 103.8 ± 14.31 , M (122.5), 75th (154.3); $U = 176.5$, $d = 0.82$] (**Figure 10B**). Thus, no noteworthy reductions in TOA were evident in ORM-10921 treated rats on days 7 or 21 in non-exposed or exposed animals vs. control (**Figure 10B**). Detailed descriptions of interquartile ranges, Cohen's d calculations, and Mann-Whitney statistics, are provided in Tables 1 – 3.

3.2.2 Closed arm behaviour

3.2.2.1 Closed arm entries (Figure 11A)

Following a mixed model analysis to examine the effects of exposure, time, and treatment on the number of closed arm entries in the EPM, a statistically significant three-way interaction between the three factors could not be demonstrated [$F(1, 56) = 0.68$, $p = 0.4$]. Moreover, neither treatment and time [$F(1, 56) = 1.89$, $p = 0.2$, exposure and time [$F(1, 56) = 0.05$, $p = 0.83$] nor exposure and treatment, $F(1, 56) = 0.000$, $p = 1.00$ demonstrated any significant interaction. Detailed descriptions of interquartile ranges, Cohen's d calculations, and Mann-Whitney statistics, are provided in Tables 1 – 3.

3.2.2.2 Time spent in the closed arms (Figure 11B)

A mixed model analysis of time in the closed arms failed to reveal a statistically significant three-way interaction between exposure, time and treatment [$F(1, 56) = 0.41$, $p = 0.52$] or significant simple two-way interactions for either treatment and time [$F(1, 56) = 3.35$, $p = 0.07$], exposure and time [$F(1, 56) = 2.776$, $p = 0.10$], or exposure and treatment, [$F(1, 56) = 1.079$, $p = 0.30$]. However, chronic saline treatment significantly increased time in the closed arms in the non-exposed cohort on post-exposure day 21 compared to day 7 [197.5 ± 22.0 , M (186.8), 25th (88.3) vs. 125.1 ± 13.8 , M (125.4), 25th (85.6); $U = 13$, $p = 0.04$, $d = 1.16$] (**Figure 11B**). However, no such trends were observed in the exposed cohort, while no differences in time in the closed arms were evident in the ORM-treated groups of both non-exposed and exposed animals. Detailed descriptions of interquartile ranges, Cohen's d calculations, and Mann-Whitney statistics, are provided in Tables 1 – 3.

3.2.3 Head dipping behaviour (Figure 12)

A mixed model analysis was used to examine the effects of exposure, time, and treatment on the number of head dipping episodes in the EPM as a measure of risk assessment and exploratory behaviour. A three-way interaction between the three factors failed to reach significance [$F(1, 56) = 0.201, p = 0.67$]. Further simple two-way interactions between treatment and time [$F(1, 56) = 0.259, p = 0.61$], exposure and time [$F(1, 56) = 0.463, p = 0.50$], and exposure and treatment [$F(1, 56) = 0.202, p = 0.655$] were found to be insignificant. However, exposed saline treated animals showed significantly reduced head dipping episodes vs. non-exposed saline treated controls on day 21 [12.00 ± 2.00 M (10.00), 75th (14.75) vs. 6.50 ± 1.375 , M (7.00), 75th (9.50), $U = 21, p = 0.03, d = 1.26$] (**Figure 12**). Moreover, ORM-10921 treatment resulted in a highly practical effect size in terms of an increase in head dipping episodes in animals of both exposure cohorts on day 21 compared to their respective saline treated controls [non-exposed: 22.0 ± 2.6 , M (22.0), 75th (32.0) vs. 12.00 ± 2.000 , M (10.00), 75th (14.75), $U = 30, p = 0.03, d = 2.42$; exposed: 21.0 ± 3.0 , M (18.5), 75th (31.5) vs. 6.5 ± 1.4 , M (7.0), 75th (9.5), $U = 53, p = 0.001, d = 4.29$] (**Figure 12**). Detailed descriptions of interquartile ranges, Cohen's d calculations, and Mann-Whitney statistics, are provided in Tables 1 – 3.

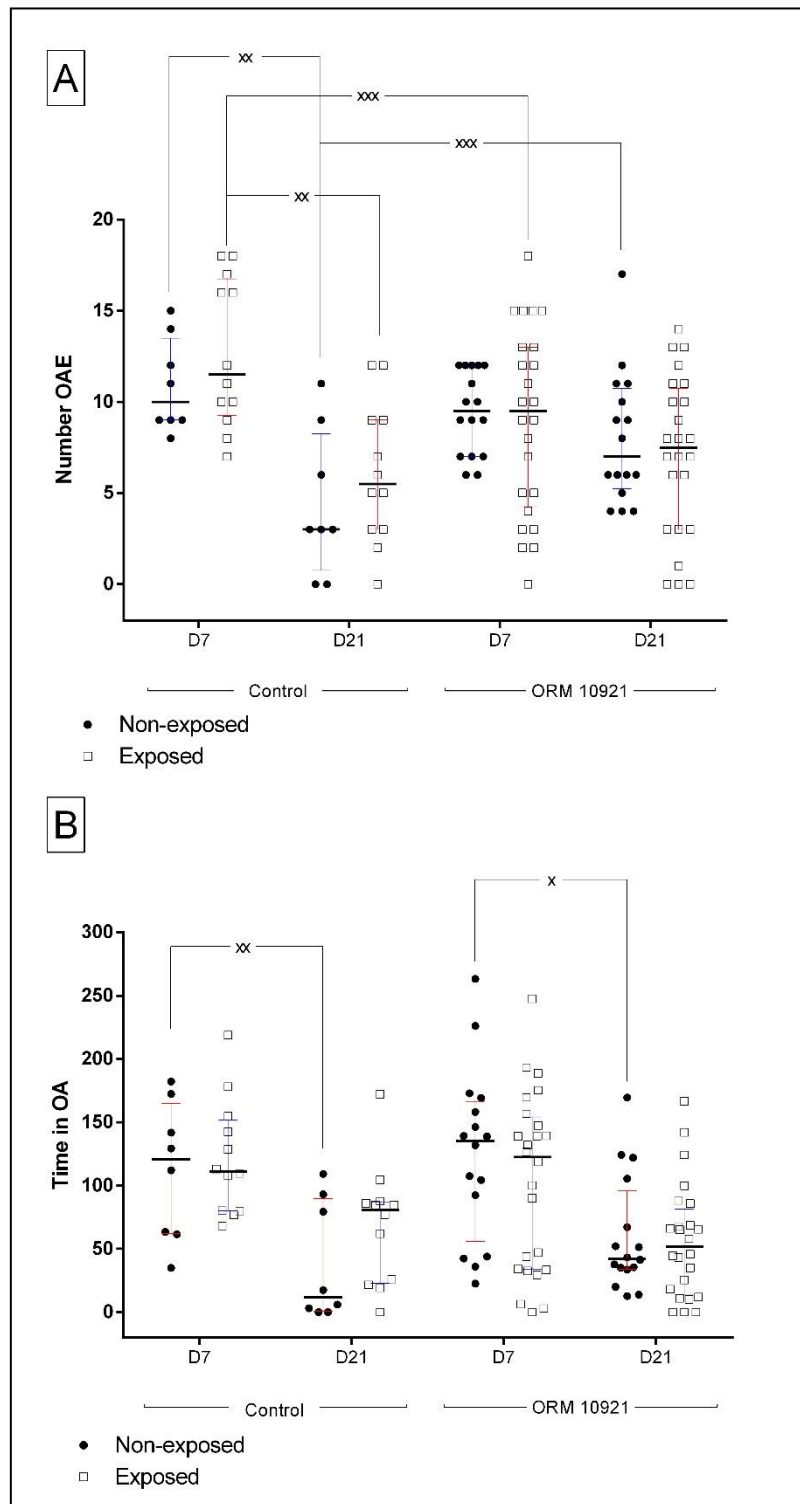


Figure 10 – Comparison of (A) number of open arm entries and (B) time spent in open arms, in rats exposed to clean or cat scented cloth, treated with either saline (control) or α_2C AR antagonist, ORM-10921. Data presented as median with interquartile range. $\times$ Cohen's d effect size; $\times d > 0.8$ $d < 1.3$; $\times \times 1.3 > d < 2.0$; $\times \times \times d > 2.0$

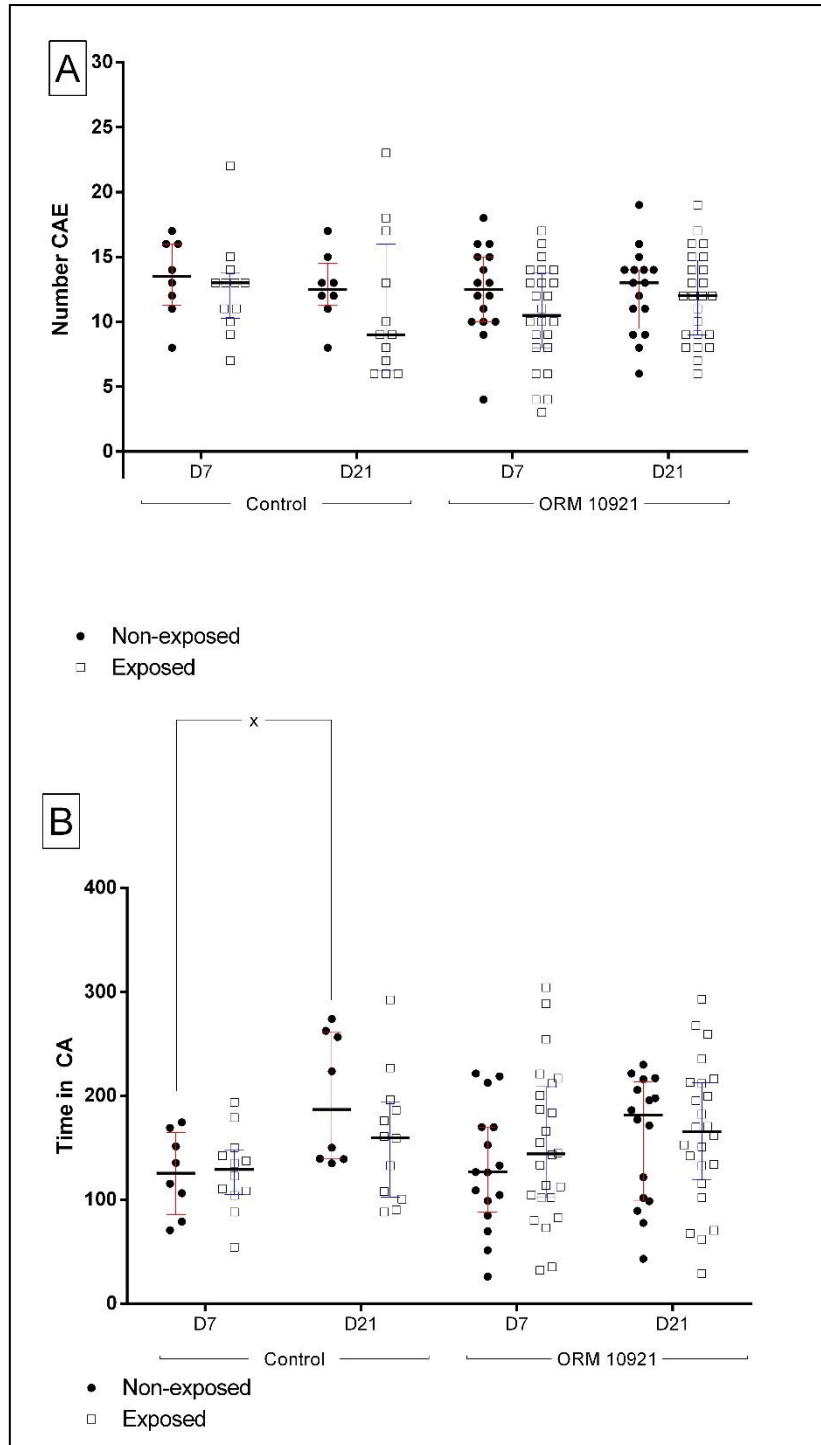


Figure 11 – Comparison of (A) number of closed arm entries and (B) time spent in closed arms, in rats exposed to clean or cat scented cloth, treated with either saline (control) or α_{2c} AR antagonist, ORM-10921. Data presented as median with interquartile range. *Cohen's d effect size; *0.8 > d < 1.3

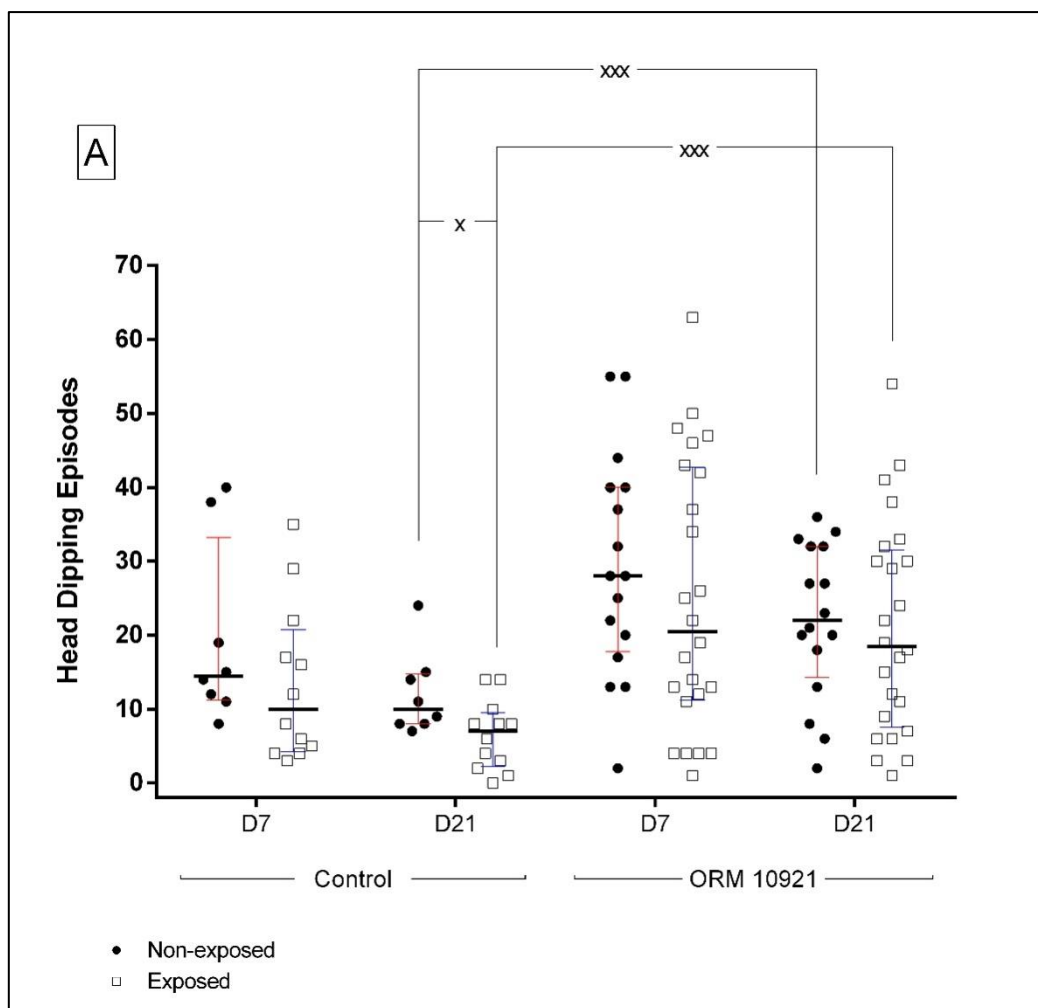


Figure 12 – Comparison of number of head dipping episodes, in rats exposed to clean or cat scented cloth, treated with either saline (control) or α_{2c} AR antagonist, ORM-10921. Data presented as median with interquartile range.

xCohen's *d* effect size; xd 0.8 > *d* < 1.3; xxxd > 2.0

Table 1: Descriptive statistics of the interquartile of distribution between-group behaviour in different parameters for immediate treatment

Comparisons	(NE: non-exposed / E: exposed)	Open arm entries 75 th	Time in open arms (s) 75 th	Closed arm entries 25 th	Time in closed arms (s) 25 th	Head dipping episodes 75 th
Saline-treated animals	NE_7	8.250	164.8	11.25	85.65	33.25
	NE_21	13.50	89.60	11.25	139.1	14.75
	E_7	16.75	151.8	10.25	105.1	20.75
	E_21	9.00	87.20	6.250	102.3	9.500
ORM 10921- treated animals	NE_7	12.00	166.5	8.00	88.30	40.00
	NE_21	10.75	95.85	9.00	99.40	32.00
	E_7	13.00	154.3	10.00	101.9	42.75
	E_21	10.75	81.50	9.500	119.6	31.50

Table 2: Descriptive statistics of Cohen's d value (x) between-group behaviour in different parameters (x: > 0.8 – 1.3; xx: 1.3 – 1.99; xxx: ≥ 2.0)

Comparison of behaviour parameters of Saline vs ORM using Cohen's d values

Comparisons	(NE: non-exposed / E: exposed; S: saline / O: ORM- 10921)	Open arm entries	Time in open arm	Closed arm entries	Time in closed arms	Head dipping episodes
Saline-treated animals	NE_7 vs. NE_21	1.62	1.32	0.22	1.16	0.56
	E_7 vs. E_21	1.64	0.95	0.42	0.52	0.51
	NE_7 vs. E_7	1.38	0.52	0.65	0.10	1.30
	NE_21 vs. E_21	1.31	1.69	1.34	1.88	1.26
ORM 10921- treated animals	NE_7 vs. NE_21	0.36	1.16	0.37	0.47	0.55
	E_7 vs. E_21	0.48	0.82	0.37	0.19	0.29
	NE_7 vs. E_7	0.47	1.64	2.04	1.57	1.45
	NE_21 vs. E_21	1.00	0.34	0.58	0.32	0.33
Saline vs. ORM 10921	S_NE_7 vs. O_NE_7	1.16	0.73	0.87	0.23	2.37
	S_NE_21 vs. O_NE_21	2.99	1.28	0.217	2.00	2.42
	S_E_7 vs. O_E_7	3.65	1.28	2.26	1.59	3.42
	S_E_21 vs. O_E_21	0.99	0.93	0.93	0.27	4.29

Table 3: Descriptive Mann-Whitney statistics for immediate treatment*Comparison of behaviour parameters of Saline vs. ORM using Mann-Whitney U*

Comparisons	(NE: non-exposed / E: exposed; S: saline / O: ORM-10921)	Open arm entries	Time in open arms	Closed arm entries	Time in closed arms	Head dipping episodes
Saline-treated animals	NE_7 vs. NE_21	7 (p=0.007)	9 (p=0.01)	26.5 (p=0.60)	13 (p=0.04)	17.5 (p=0.13)
	E_7 vs. E_21	-6 (p=0.001)	32 (p=0.1)	48 (p=0.17)	52.5 (p=0.27)	43.5 (p=0.12)
	NE_7 vs. E_7	35 (p=0.32)	43 (p=0.73)	36.5 (p=0.38)	47 (p=0.96)	31 (p=0.20)
	NE_21 vs. E_21	35.5 (p=0.34)	31 (p=0.20)	32 (p=0.22)	33 (p=0.27)	21 (p=0.03)
		-2.5 (p=0.07)	55 (p=0.005)	125 (p=0.91)	94.5 (p=0.21)	91.5 (p=0.17)
ORM 10921- treated animals	E_7 vs. E_21	-2 (p=0.17)	176.5 (p=0.02)	238.5 (p=0.31)	258 (p=0.54)	253.5 (p=0.48)
	NE_7 vs. E_7	189 (p=0.93)	161 (p=0.40)	137.5 (p=0.13)	162 (p=0.41)	158 (p=0.35)
	NE_21 vs. E_21	180 (p=0.74)	184 (p=0.83)	174.5 (p=0.63)	190 (p=0.96)	173 (p=0.60)
Saline vs. ORM 10921	S_NE_7 vs. O_NE_7	47.5 (p=0.32)	60 (p=0.83)	52 (p=0.47)	63 (p=0.97)	36 (p=0.08)
	S_NE_21 vs. O_NE_21	29 (p=0.03)	39 (p=0.13)	64 (p=0.99)	41 (p=0.17)	30 (p=0.03)
	S_E_7 vs. O_E_7	86.5 (p=0.05)	127 (p=0.58)	108.5 (p=0.23)	118 (p=0.39)	92.5 (p=0.08)
	S_E_21 vs. O_E_21	121 (p=0.44)	114 (p=0.33)	112.5 (p=0.29)	133.5 (p=0.73)	53 (p=0.001)

3.3 Correlational analyses between corticosterone concentrations and behavioural manifestations

Corticosterone concentrations were measured only on day 21 post-exposure, and subsequently correlated with behavioural manifestations on day 7 and day 21 post-PSE. This was done in order to determine whether behaviour on day 7 could provide a prediction of the end-point corticosterone concentrations measured and/or whether corticosterone concentration will only demonstrate an association with end-point behaviour on day 21.

3.3.1 Corticosterone concentrations vs. the number of open arm entries (Figure 13)

A Spearman's rank order correlation was used to assess the relationship between corticosterone concentration on day 21 and the number of open arm entries made by saline-treated rats on day 7 and 21. Preliminary analyses showed the relationship to be linear with one of the variables not being distributed normally, as assessed by the Shapiro-Wilks test ($p > 0.05$). A small negative correlation between corticosterone concentration and open arm entries was demonstrated on day 7 ($R^2 = 0.07$, $p = 0.35$), but not for day 21 ($R^2 = 0.0004$, $p = 0.95$) (**Figure 13A**).

A Pearson's correlation was run to assess the relationship between corticosterone concentration on day 21 and the number of open arm entries on day 7 and day 21 in ORM-10921 treated rats. Preliminary analyses showed the relationship to be linear with both variables normally distributed, as assessed by Shapiro-Wilks test ($p > 0.05$). As opposed to saline-treated animals, a moderate positive correlation between corticosterone concentration and number of open arm entries was demonstrated on day 7 ($R^2 = 0.1553$, $p = 0.006$), but not day 21 ($R^2 = 0.1101$, $p = 0.1132$) (**Figure 13B**). Corticosterone concentration explained 15% of the variation in the number of open arm entries on day 7 and 11% on day 21.

3.3.2 Corticosterone concentrations vs. the time spent in the open arm (Figure 14)

A Pearson's correlation was run to assess the relationship between corticosterone concentration and the time spent in open arm on day 7 and day 21 in saline treated rats. Preliminary analyses showed the relationship to be linear with both variables normally distributed, as assessed by Shapiro-Wilks test ($p > 0.05$) and there were no outliers. There was a small positive correlation on day 7 between concentration and time spent in open arms ($R^2 = 0.02$, $p = 0.65$) (**Figure 14A**).

A Pearson's correlation assessing the relationship between corticosterone concentration and the time spent in open arm on days 7 and day 21 in ORM-10921 treated rats revealed a small positive correlation between endpoint corticosterone concentration and time spent in the open arms on day 7 ($R^2 = 0.03$, $p = 0.34$), but not on day 21 ($R^2 = 0.11$, $p = 0.10$) (**Figure 14B**).

Corticosterone concentrations vs. the number of closed arm entries (Figure 15)

A Spearman's rank order correlation was used to access the relationship between corticosterone concentration and number of closed arm entries on day 7 and 21 in saline treated rats. Preliminary analyses showed the relationship to be linear with one of the variables not being distributed normally, as assessed by Shapiro-Wilks test ($p > 0.05$). No correlation could be demonstrated (**Figure 15A**).

A Pearson's correlation also failed to reveal any significant association between corticosterone concentration and closed arm entries in ORM-10921 treated rats on with respect to both day 7 and day 21 (**Figure 15B**).

3.3.3 *Corticosterone concentrations vs. the time in closed arms (Figure 16)*

Again, no significant correlations between corticosterone concentrations and time spent in the closed arms could be demonstrated for either saline or ORM-10921 treated rats in either the non-exposed or the exposed cohorts (**Figure 16A and B**).

3.3.4 *Corticosterone concentrations vs. the number head dipping episodes (Figure 17)*

Also with respect to correlational analyses of corticosterone analyses and the number of head-dipping episodes, no significant correlations could be demonstrated (**Figure 17A and B**).

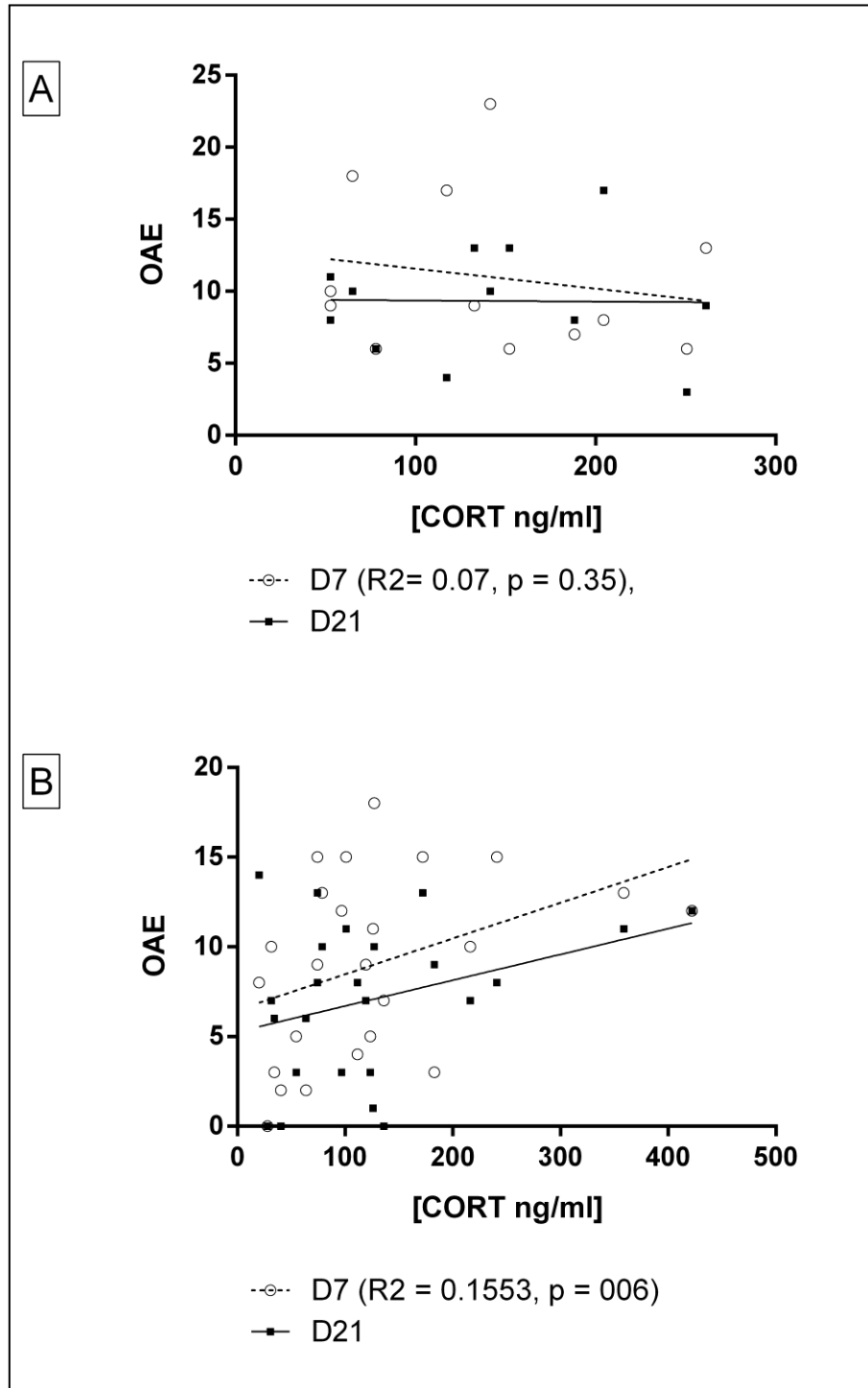


Figure 13 – The effect of PSE on plasma corticosterone concentrations vs. number of open arm entries in saline and ORM-10921-treated animals. A) Spearman's rank order correlation (saline treatment); B) Pearson's correlation (ORM-10921 treatment); OAE = open arm entries

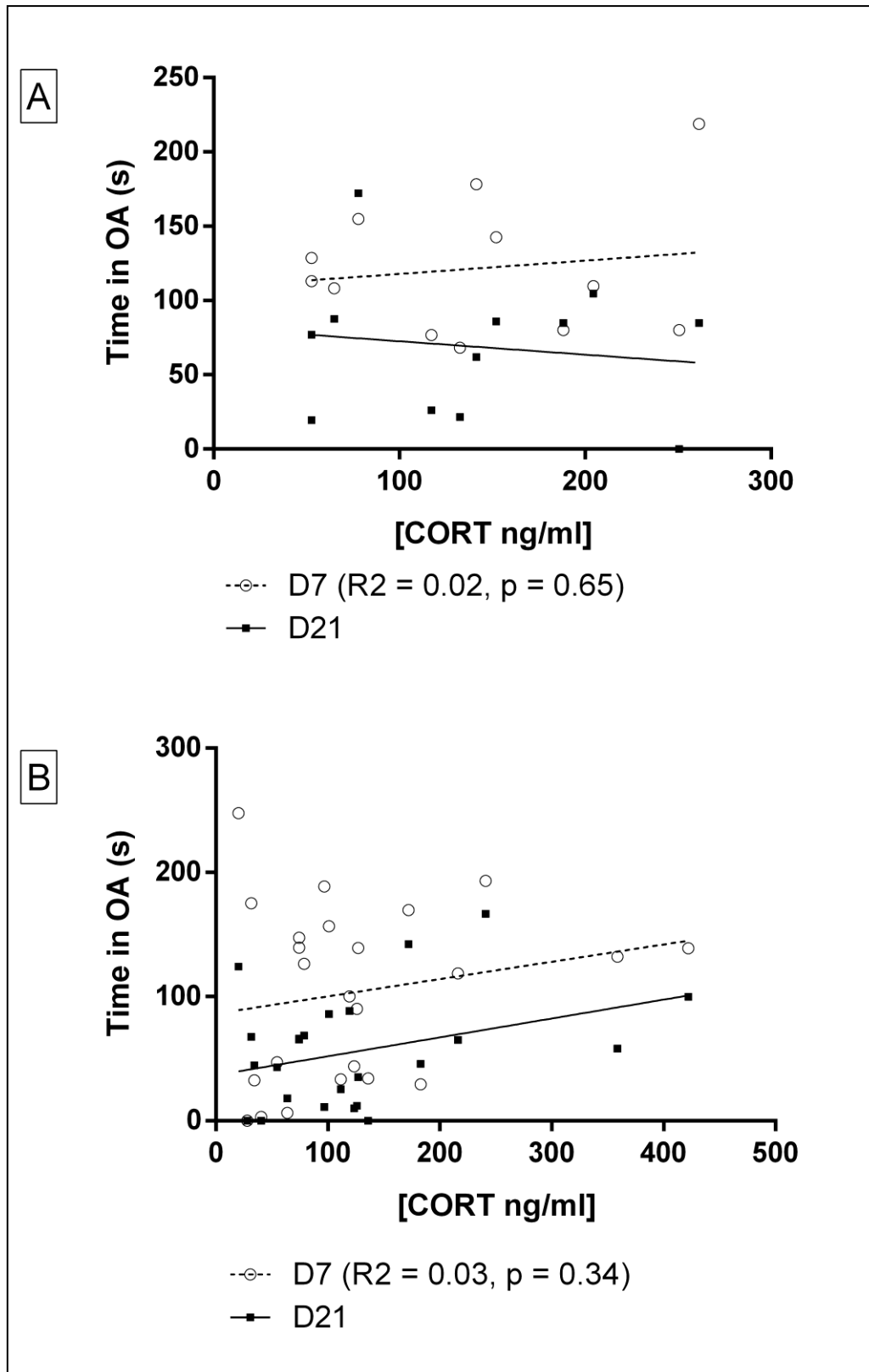


Figure 14 – The effect of PSE on corticosterone plasma concentrations vs. time spent in the open arms in saline and ORM-10921-treated animals. A) Spearman's rank order correlation (saline treatment) on day 7 ($R^2 = 0.02$, $p = 0.65$); B) Pearson's correlation (ORM-10921 treatment) day 7 ($R^2 = 0.03$, $p = 0.34$), and day 21 ($R^2 = 0.11$, $p = 0.10$); OA = open arms

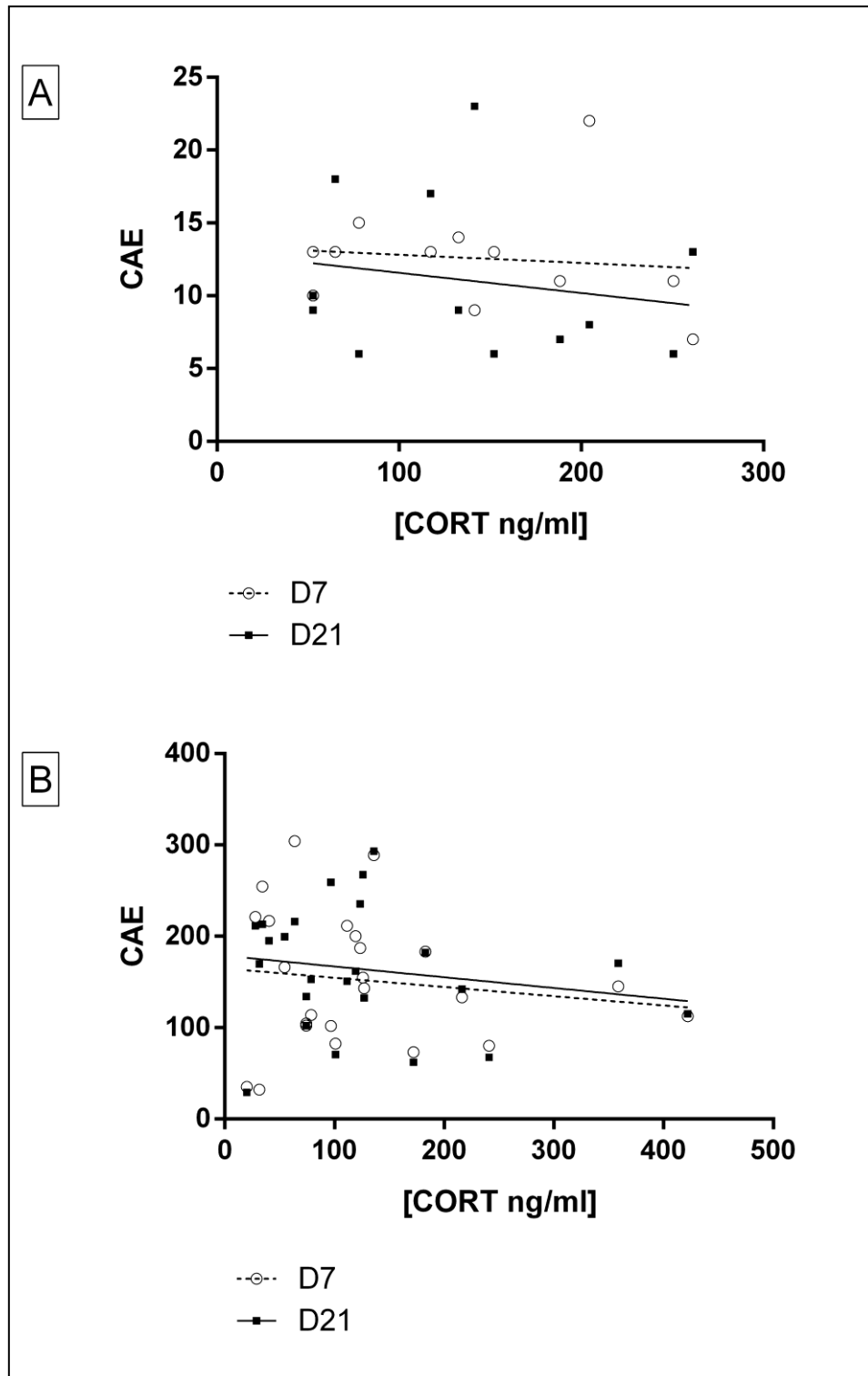


Figure 15 – The effect of PSE on corticosterone plasma concentrations vs. number of closed arm entries in saline and ORM-10921-treated animals. A) Spearman's rank order correlation (saline treatment); B) Pearson's correlation (ORM-10921 treatment); CAE= Closed arms

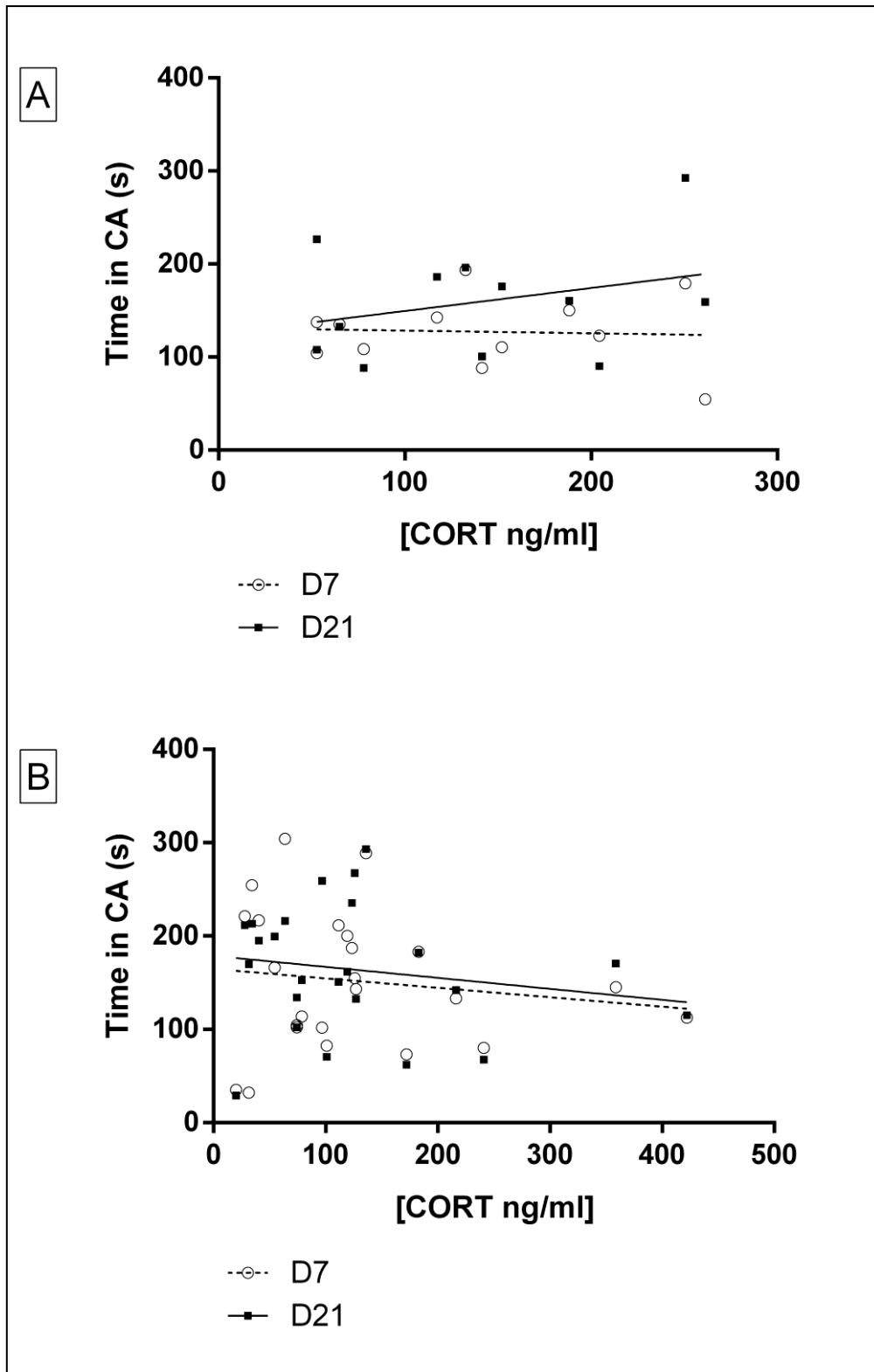


Figure 16 – The effect of PSE on corticosterone plasma concentrations vs. time spent in the closed arms in saline and ORM-10921-treated animals. A) Spearman's rank order correlation (saline treatment); B) Pearson's correlation (ORM-10921 treatment). CA= Closed arms

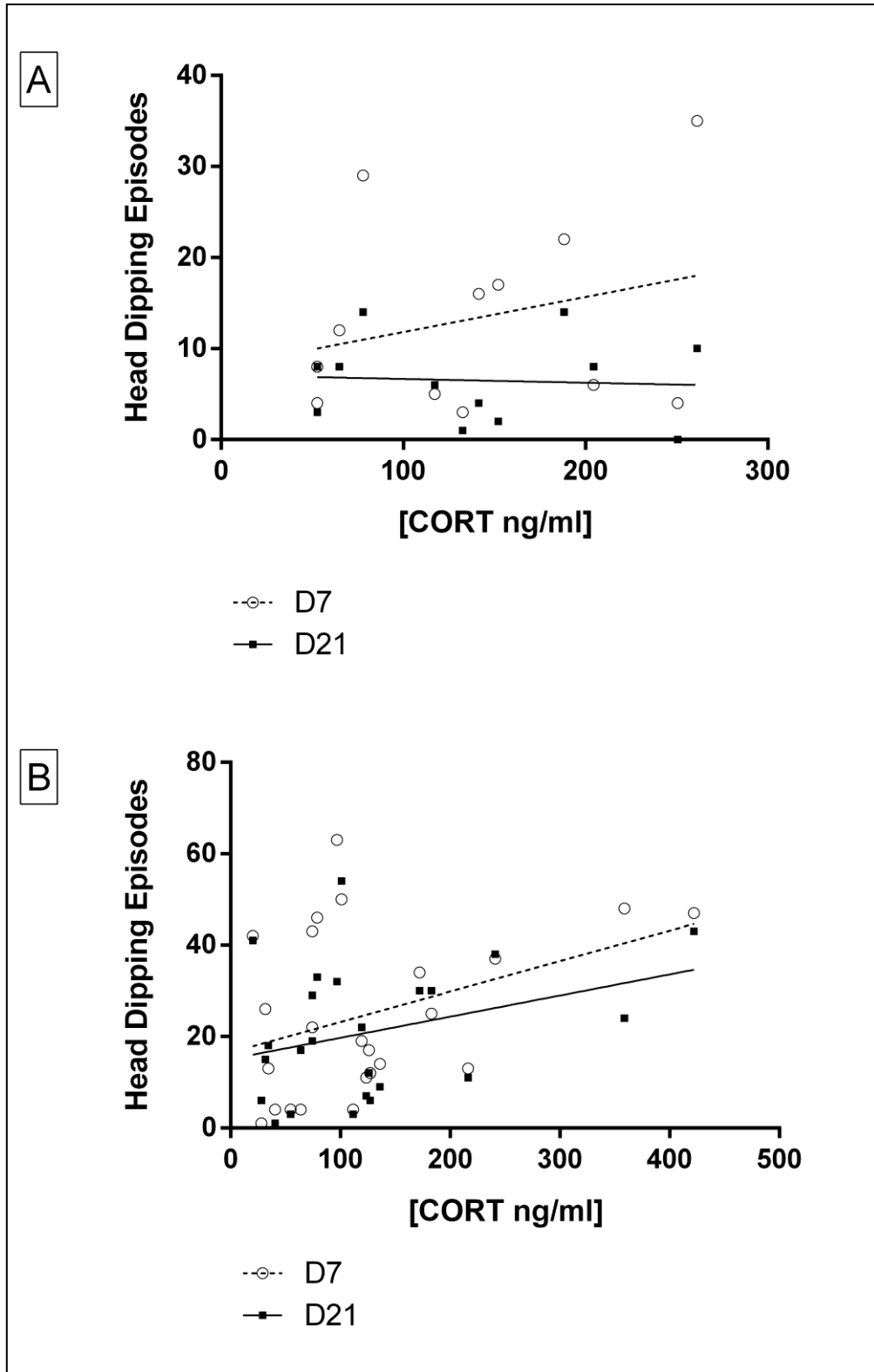


Figure 17 – The effect of PSE on corticosterone plasma concentrations vs. number of head-dipping episodes made by saline and ORM-10921-treated animals. A) Spearman's rank order correlation (saline treatment); B) Pearson's correlation (ORM-10921 treatment).

4 Discussion

The present investigation resulted in several significant findings related to the behavioural aspects of the PSE model of PTSD and its response to immediate, and chronic ORM-10921 interference. First, a single 10-minute exposure to predator scent, but not clean cloth, elicited a significant anxiety-like response during exposure, as previously described (Adamec and Shallow 1993; Adamec *et al* 1997; Cohen 1997; 1999), as evident by the increase in the number of faecal boli in the exposed compared to the non-exposed cohort (Figure 4). Second, PSE was associated with a significant increase in delayed onset post-exposure anxiety-like manifestations 7 days later, as evinced by significant reductions in the number of open arm entries (Figure 5) and time spent in the open arms (Figure 7), as well as a significant increase in the time spent in the closed arms (Figure 8) vs. non-exposed animals. Third, exposed animals presented with significantly less risk-taking behaviour, as evinced by a reduction in the number of head dipping episodes (Figure 9) compared to the non-exposed cohort. Fourth, these changes also only occurred in approximately 25% of the exposed population; this indicated by at least one quarter of PSE animals demonstrated demonstrating more anxiety-like behaviour in the EPM, compared to *any* of the non-exposed individuals with respect to the number of entries into the open and the number of head dipping episodes, as well as time spent in the open and closed arms. That at least 25% of PSE animals demonstrated significantly more anxiety-like behaviour vs. non-PSE individuals is in agreement with the typical risk: resilience ratio described in clinical studies in PTSD (Breslau *et al*, 1991; Shalev *et al* 2000 ;Cohen *et al.*, 2004), and is in agreement with previous studies in rats (Cohen *et al* 2003,2006). Fifth, chronic administration of ORM-10921 (0.3mg /kg x 21 days), starting immediately after PSE, significantly reduced PSE-mediated behavioural disruptions, as demonstrated by the large effect size differences between the ORM and saline treated cohorts (non-exposed: $d = 2.99$; exposed: $d = 3.6$), with saline treated cohorts presenting with less open arm entries over time compared to ORM-10921 treated animals (Figure 8A). Here ORM-10921 reversed the decrease in open arms entries vs. saline-treated PSE animals (Figure 10 A). Although the number of closed arm entries for both groups were high (Figure 11A), this could be due to natural tendency of animals to rather seek protection, than entering open unprotected spaces (Handley & Mithani, 1984). However, ORM-10921 tended to prevent a prolonged presence in the closed arms (Figure 11B), as can be seen with the distribution of the interquartile ranges and smaller effect sizes compared to saline treated cohorts (Tables 1, 2 and 3). Therefore, an anxiolytic profile is evident in the behaviour of ORM-10921 treated animals. Sixth, ORM-

10921 reversed a reduction in risk assessment and exploratory behaviour in PSE animals compared to saline treated PSE animals (Figure 12). Indeed, ORM-10921 induced an increase of large effect in the number of head dipping episodes vs. PSE animals treated with saline (non-exposed $d = 2.42$ and exposed $d = 4.29$; Figure 12; Table 2). These data not only confirm the efficacy of ORM-10921 to reverse PTSD-like aversive behaviour but suggest that the α_{2C} AR is causally linked to the genesis of anxiety in these animals, and by implication in PTSD.

Considering the neuroendocrine changes elicited by chronic ORM administration, it did not affect plasma corticosterone in either PSE or non-PSE rats, although a trend towards hypocortisolemia was evident (Figure 13-17). However, upon closer scrutiny, ORM-10921 treated animals were characterised by an increase in plasma corticosterone and an associated lowering of anxiety (Figure 13). Indeed, ORM-10921 resulted in a large trend to increase corticosterone and to increase open arm entries and time spent in the open arms (i.e. less anxious). This was observed on day 7 ($R^2 = 0.1553$, $p = 0.006$) but not on day 21 with respect to open arm entries (Figure 13A), and time spent in the open arms on day 7 ($R^2 = 0.03$, $p = 0.34$) but not on day 21 (Figure 13B), compared to saline treated cohorts. Thus, behavioural severity on day 7 predicts a treatment response with regards to adaptations in corticosterone concentrations following ORM-10921 treatment, since the more anxious the animals are on day 7 the worse their response to treatment would be with regards to corticosterone concentration. Earlier studies by Cohen and colleagues (2000) have found that lower levels of plasma corticosterone and prolactin in ketoconazole treated and PSE exposed rats were accompanied by lower anxiety compared to untreated exposed rats. This is essentially opposite to the findings described here, although the effects of chronic stress on corticosterone and ACTH secretion may vary depending on the experimental paradigm (Cohen *et al.*, 2000). It has been reported that an adaptation to chronic stress may occur, which results in decreased plasma ACTH and corticosterone levels compared with levels following a single acute stressor which evokes an increase in glucocorticoids (Kant *et al.*, 1985; Kant *et al.*, 1987). It may thus be that the current CORT data reflects that of a more acute stressor and where exaggerated negative feedback on the HPA-axis has not yet developed, as is typical of PTSD.

Since hypercortisolemia is elicited to support coping with an aversive/stressful condition, stress resilient individuals may show benefit from such an endocrine response by having a lower level of anxiety, indicative of better coping strategies under stress (Yehuda, 2009). On

the other hand, an at-risk individual would respond in a maladaptive manner, presenting with the typical manifestations of anxiety. In fact, cortisol is responsible for containing the catecholamine system during stress; it assists in lowering the high levels of adrenaline that are released during fight or flight (Yehuda, 2009), so that hypercortisolemia is not necessarily a detrimental consequence; rather it is how this state persists over time and whether it becomes maladaptive. The worse scenario would be progression to increased HPA-axis suppression with hypocortisolemia, which in itself may also be associated with anxiety, as has been demonstrated using the stress-restress model of PTSD (Harvey *et al.*, 2006). Therefore, in our hands, the PSE model demonstrates robust face validity for PTSD, as demonstrated by the evidence of anxiety-like behaviour as noted in the EPM, hypocortisolemia, as well as the positive correlation between elevated plasma corticosterone and markers of reduced anxiety. Therefore, chronic treatment with ORM-10921, a novel α_{2C} AR antagonist, abolished the chronic behavioural effects of acute exposure to trauma-like interference. The time frame in which treatment was administered (1 hour after stress exposure) conforms to the time frame within which fear memory consolidation takes place (Cohen *et al.*, 2006). The time at which the effect was assessed was sufficiently distant from the stimulus as to suggest that the effect may well be mediated by fear conditioning to strengthen and consolidate fearful memories related to the trauma. In fact, this idea is supported by previous studies in which anisomycin, a protein synthesis inhibitor, was effective in attenuating behavioural fear responses when administered within 1 hour after PSE but not when administered later on (Cohen *et al.*, 2006). In fact, pharmacotherapy targeting fear conditioning may be clinically useful in the treatment of stress-induced disorders as mentioned earlier (Orr *et al.*, 2000). A number of agents that modulate fear conditioning processes, such as NMDA receptor antagonists (Charney, 2004), Ca^{2+} channel blockers (Charney, 2004), opioid agonists (Saxe *et al.*, 2001) and adrenergic receptor antagonists (Pitman *et al.*, 2002; Vaiva *et al.*, 2003) may be useful to reduce the impact and intensity of recently acquired fear memory. It is in this class that ORM-10921 falls. Thus, results in this model implicate a mutual interaction between corticosterone and NA in the pathogenesis of chronic anxiety following acute psychological trauma. More specifically, the role of NA seems to involve the α_{2C} AR.

Looking closer at the possible mechanism of action of ORM-10921 in the current scenario, the biological actions of the α_{2C} -receptor must be elaborated on. Activation of the noradrenergic system enhances memory function since noradrenalin increases neural firing in the hippocampus, suggesting a possible role in the enhancement of memory storage of

contextual memory related to the trauma (Madison & Nicoll, 1982). Noradrenergic brain systems are also involved in the neural mechanisms of fear conditioning (Cahill, 2000), fear extinction as well as sensitization, which are relevant to the animal models of stress, panic disorders and PTSD (McGaugh *et al.*, 2002; McGaugh, 2004). Fear conditioning is underpinned by the release of noradrenalin in the hippocampus and amygdala (Madison & Nicoll, 1982; Soeter & Kindt, 2011), whereby increased NA in the amygdala is crucial for memory consolidation. Emotionally aroused noradrenergic activation of the BLA has been shown to strengthen memory consolidation in other brain regions too, such as the hippocampus (McGaugh, 2004). These processes are highly dependent on activation of α_2 and β AR in the amygdala, with antagonism of these receptors inhibiting of memory consolidation and agonism enhancing consolidation (Liang *et al.*, 1986; Liang *et al.*, 1990; Ferry *et al.*, 1999b; Ferry *et al.*, 1999a). This is supported by the clinical efficacy of the $\beta_{1/2}$ AR antagonist, propranolol, in patients with PTSD (Friedman, 1997; Vaiva *et al.*, 2003). However, despite some evidence for efficacy as a prophylactic agent in patients at risk of developing posttraumatic stress disorder (PTSD) (Vaiva *et al.*, 2003; Dębiec & Ledoux, 2004; Schwabe *et al.*, 2014), its clinical value is criticised by many (Giustino *et al.*, 2016). This may suggest that specifically targeting other noradrenergic receptors, like the α_{2c} AR, may be a more promising avenue of investigation.

α_{2c} AR messenger RNA demonstrates significant expression in the amygdaloid complex, hypothalamus, olfactory system and the hippocampal formation (Wang *et al.*, 1996), while also populating the ventral and dorsal striatum and the cortex (Uys *et al.*, 2017). It is therefore likely that the anxiolytic actions of ORM-10921 described in this work are related to how the above-mentioned brain regions are involved in the stress response, in particularly memory formation, recall and extinction of a fearful event, or the regulation of the HPA-axis. The cortex and hippocampus may be especially relevant. Re-exposure of an animal with a history of chronic stress potentially results in the release of noradrenalin in the hippocampus thus possibly representing the mechanism by which exposure to stressful environmental stimuli is associated with the recall of abnormal traumatic memories (Madison & Nicoll, 1982; Mather *et al.*, 2015). Therefore, an α_{2c} antagonist may interfere with the consolidation of short term memory into long-term memory (Liu & Zhou, 2015).

The presence of the α_{2c} AR in the hypothalamus may allow ORM-10921 to interfere with corticosterone synthesis and release, especially stress-induced CORT release from the adrenal glands (Wang *et al.*, 1996); however, it is unusual then that ORM-10921 did not

notably affect plasma corticosterone in PSE or non-PSE animals. Since the re-experiencing cluster of symptoms in PTSD is thought to be due to inadequate control over the fearful memory traces formed at the trauma, this related to increased HPA-axis negative feedback and hypocortisolemia (Yehuda *et al.*, 1991; Liberzon *et al.*, 1999; Yehuda, 2009) and well as sustained hyperadrenergic activity in the aftermath of the trauma, this work establishes targeted antagonism of the α_{2C} AR with ORM-10921 as a possible treatment modality in PTSD, at least in regard to suppressing late onset anxiety when administered immediately post-trauma. In fact, the immediate post-PSE inhibition of the α_{2C} AR will promote the release of NA (Sallinen *et al* 2007). This seems contradictory to the role of NA in the coping response, where excessive NA is more commonly associated with anxiety (Sallinen *et al* 2007), increased NA has also been linked to an anxiolytic action, probably via the action of NA on neighbouring 5HTergic, GABAergic and glutamatergic neurons (Harvey and Slabbert, 2014). Human clinical drug trials have demonstrated the efficacy of anti-glutamatergic drugs for the treatment of obsessive-compulsive disorder, posttraumatic stress disorder, generalized anxiety disorder, and social phobia (Cortese & Phan, 2005). In this regard, and with respect the work presented here, it may be possible that, contradictory to previous findings (Sallinen *et al* 2013), ORM-10921 has been found to alleviate a hyperglutamatergic state, proposing a possible role for ORM-10921 to alleviate anxiety like behaviour in PTSD. However, it must be stated that the effects of ORM-10921 in the current investigation, may be brain region and stress-paradigm specific. Further, studies have shown that in the brain, α_{2A} - and α_{2C} -receptors inhibit dopamine release in basal ganglia (Bücheler *et al.*, 2002) as well as serotonin secretion in mouse hippocampus and brain cortex (Scheibner *et al.*, 2001). Looking more closely at the neuroendocrine data, absence of the α_{2C} AR (e.g. in the α_{2C} -KO mouse), thus akin to α_{2C} AR antagonism, has been associated with a decrease in corticosterone levels following repeated stress (Sallinen *et al* 2007). Moreover, α_{2C} -KO mice also demonstrate protective effects against stress (Sallinen *et al* 2007). The actions of ORM-10921 in this study relating to the corticosterone data referred to above, seems to contradict our own findings that demonstrate a time-dependent increase in corticosterone concentration, associated with anxiolytic-like behaviour. However, differences in the type of stressor, onset and duration of treatment, may interfere uniquely in different investigations.

To conclude, this study has provided valuable new information on the role of ORM-10921 in PTSD, demonstrating significant anxiolytic effects and showing therapeutic potential in

preventing the development of acute stress syndrome into full-blown PTSD. Moreover, these findings have contributed significantly to current knowledge regarding the neurobiology underlying PTSD and anxiety likebehaviour, and how these processes can be pharmacologically targeted to alter the course of PTSD. The study has not only emphasized the central role of NA in PTSD, in particular the α_{2C} AR. The study has established the predictive validity of the PSE model in our laboratory where it can now be applied in future exploratory studies into stress and anxiety disorders. Further studies employing these novel agents in the treatment of anxiety disorders, such as PTSD, are encouraged.

However, some limitations to this study are worth noting:

- The fact that group numbers were not the same could possibly have prevented statistical significance in certain key analyses. However this was unavoidable due to ethical constraints, the time frame of the study and the number of animals available.
- The fact that a dose response was not conducted in this study could possibly be a limitation as the novel compound, ORM-10921, has never been tested in animal models of PTSD before. However, based on previous animal studies in our laboratory using ORM-10921 and that established its antidepressant activity at a dose of 0.3 mg/kg (Uys *et al* 2017), we are confident that the dosage used in this study falls within the maximally effective dose range. Although a 3-tiereddose ranging study would have been desirable, it was beyond the ethical framework, time line and budget for the study.
- Another limitation could possibly be that treatment response with ORM-10921 is dependent on the time of administration, either being initiated immediately (day 0) or distal (day 7) to the traumatic event. This confounding variable has been addressed in the addendum, where we undertook delayed ORM-10921 treatment one week post-trauma to assess whether ORM-10921 treatment showed a better, similar or weakened response when initiated distal to the traumatic event, as opposed to immediately post-trauma as described here.
- The duration of treatment for the 7 days post-trauma group was another shortcoming as we did not have a group of animals that were treated from day 7 for 21 days, as done with the immediate post trauma treatment group. This discrepancy complicates interpretation of the results across the two treatment periods. However, that PTSD-like manifestations also demonstrate time-dependent modification, necessitates future

investigations employing behavioural analyses for a lengthened period of time, to accommodate both treatment modalities, i.e. immediately post PSE for 28 days, and 7 days post PSE for 21 days, while comparing behavioural manifestations on the same developmental timeframe.

- The natural progression of stress-related changes was also not looked at since we did not include a group of animals only exposed (not treated) and measured in the EPM on day 0, 7 and 21. However this was not part of the study and we aimed 1) to validate a potentially useful model of PTSD (which we have showed in the validation with groups I and II), and 2) to determine the response of immediate interference with either ORM or saline in this model.
- We further did not employ a positive control in the present investigation. While saline was employed as an appropriate negative control that forms the foundation of the current study, we originally intended venlafaxine as a positive control. Due to time restrictions and the less than foreseen availability of animals, this was unfortunately not possible in the end.
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5 Conclusion

This article has investigated the role of the α_{2C} AR in a PSE model of PTSD, with specific focus on anxiety and alterations in the HPA-axis. PSE evoked significant anxiety immediately, on day 7 and day 21 post-PSE in a quarter of individuals only, suggesting a sustained anxiogenic state. Although PSE induced a trend towards lowered plasma corticosterone levels, this was not significant. Further, this work has considered the anxiolytic effects of the selective α_{2C} AR antagonist, ORM-10921, in the model. By focusing on immediate post exposure administration, ORM-10921 demonstrated significant anxiolytic effects, thus showing therapeutic potential in preventing the development of acute stress syndrome into full-blown PTSD. Further studies employing these novel agents in the treatment of anxiety disorders, such as PTSD, are encouraged, and will further our understanding of the role of α_{2C} AR in such disorders, as its viability as a therapeutic target.

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4 CONCLUSION

Posttraumatic stress disorder (PTSD) is a psychiatric disorder that can manifest following the experience or witnessing of a life-threatening event, such as military combat, natural disasters, terrorist incidents, serious accidents, or physical or sexual assault during child- or adulthood (American Psychiatric Association, 2013). Although most survivors of a traumatic event recover over time, approximately 30% of victims will go on to develop full-blown PTSD (Nemeroff *et al.*, 2006). The disorder presents with a symptom triad consisting of re-experiencing (e.g. flashbacks, nightmares, and intrusive thoughts), hyperarousal (e.g. hypervigilance, exaggerated startle response, sleep disturbances) and avoidance symptoms and emotional numbing (avoiding places, events or people reminiscent of the trauma (American Psychiatric Association, 2013). A number of dysfunctional neurobiological pathways are implicated in the pathology underlying these symptom clusters, with the noradrenergic system playing a very prominent role (Morgan *et al.*, 1993; Southwick *et al.*, 1993; Geraciotti Jr *et al.*, 2001; Pitman *et al.*, 2002; Roozendaal *et al.*, 2004).

Activation of the noradrenergic system, particularly in the hippocampus and amygdala, plays an important role in the enhancement of fear memory as well as the storage of contextual memory of an adverse event (Madison & Nicoll, 1982). Noradrenergic systems in the brain are involved in the neural mechanisms of fear conditioning, extinction as well as sensitization, which are relevant to the animal models of stress, panic disorders and PTSD (Cahill, 2000). Re-exposure of an animal with a history of chronic stress results in a potential release of noradrenalin in the hippocampus, thus representing the mechanism by which exposure to stressful stimuli in the environment may be associated with the recall of abnormal traumatic memories (Madison & Nicoll, 1982). Differential roles for the various subtypes of α_2 ARs, namely α_{2C} , α_{2B} and α_{2C} ARs, have been recognised in tasks requiring cognitive processes (Sallinen *et al.*, 1999; Schramm *et al.*, 2001; Krystal & Neumeister, 2009), and to have therapeutic potential in several neuropsychiatric disorders that present with disturbances in cognition and memory, such as schizophrenia, depression and Alzheimer's disease (Uys *et al.*, 2017). Moreover, the amygdala has a high density of α_2 -receptors and selective blocking or activation of these receptors is expected to modulate memory storage (Ferry & McGaugh, 2008). 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

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may determine symptom development on the one hand and response to secondary pharmacological intervention on the other.

Much effort has focused on developing animal models to help study the pathophysiology of PTSD. The traumatic events that precipitate PTSD in humans often involve potentially life-threatening physical harm. Similarly, many animal models of PTSD use physical stressors (Bonne *et al.*, 2004) such as inescapable foot shocks, underwater/forced swim paradigms, immobilization/restraint stress, or a combination of multiple stressors (Bonne *et al.*, 2004). Although many of the physical stressor models reviewed above has a “psychogenic” component, they primarily involve physical pain or discomfort. In contrast, the predator scent exposure (PSE) model (Cohen *et al.*, 2000; Cohen *et al.*, 2006) involves threat, but usually no pain. In these models, rodents are exposed to species-relevant predators (predator stress) or their odour (predator threat; Blanchard and Blanchard, 1988; Dielenberg and McGregor, 2001), leading to the development of long-lasting (3 weeks or more) anxiety-like manifestations. The PSE model thus resembles a natural paradigm for inducing psychogenic stress (Adamec *et al.*, 1997). It has ethological relevance as it comprises an intense traumatic experience and results in long term changes in behavioural, autonomic and hormonal responses in rats that correlate with the symptoms of PTSD in humans (Cohen *et al.*, 2003).

Building on this foundation, the aim of the current study was to validate the PSE model in our laboratory and to determine if male Wistar rats will demonstrate different levels of anxiety akin to maladaptation and well-adaptation following PSE, as observed in a well-validated behavioural paradigm to assess anxiety, i.e. the elevated plus maze (EPM). Further we aimed to establish its application as a valid framework for novel drug discovery. The current investigation has strengthened the face, construct and predictive validities of the PSE model of post-traumatic stress related anxiety in rats by demonstrating significant anxiety in PSE vs. non-PSE animals (Chapter 3). A single 10-minute exposure to the scent of a domestic cat induced an increase in faecal boli during PSE exposure (indicative of anxiety) as well as significantly increased anxiety behaviour in rats in the EPM 7 days later, congruent with previous studies by Adamec and Shallow (1993), Adamec *et al.* (1997) and Cohen *et al.* (1999). Exposed animals also presented with significantly less risk-taking behaviour, as evident in the reduction of the number of head dipping episodes compared to the non-exposed cohort. Of relevance for an animal model of clinical PTSD, a condition that does not transpire in all trauma-exposed individuals, our data clearly demonstrate that at least one quarter of PSE animals demonstrated significantly more anxiety-like behaviour in the EPM, compared to *any*

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of the non-exposed individuals. This has been demonstrated with respect to the number of entries into the open arms (OAE) and head dipping episodes, as well as time spent in the open (sOA) and closed arms (sCA). That at least 25% of PSE animals demonstrated significantly more anxiety-like behaviour vs. non-PSE individuals is in agreement with the typical risk: resilience ratio described in clinical studies in PTSD (Breslau *et al.*, 1991; Cohen *et al.*, 2000; Shalev, 2000; Cohen *et al.*, 2004), and is in agreement with previous studies in rats (Cohen *et al.*, 2003; Cohen *et al.*, 2006).

Given evidence indicating altered plasma cortisol in patients with PTSD, the second objective of the study was to determine if maladapted rats present with altered corticosterone concentrations like that observed in clinical PTSD, when compared to well-adapted animals. Clinical PTSD is most often associated with hypocortisolemia (Yehuda *et al.*, 1991; Liberzon *et al.*, 1999; Yehuda, 2009), suggested to be due to an exaggerated HPA-axis negative feedback (Yehuda *et al.*, 1991; Liberzon *et al.*, 1999; Yehuda, 2009). Here we found a trend that PSE maladaptive rats present with lower levels of corticosterone compared to well-adaptive PSE rats. Hypocortisolemia in PTSD has been purported to explain the lack of containment of the stress response in chronic PTSD, leading to an ongoing hyperadrenergic response and its adverse effect on fear memory and other cognitive processes. Considering the response of trauma-induced modifications in corticosterone concentrations, ORM-10921 administered chronically after PSE did not affect plasma corticosterone in either PSE or non-PSE rats, although a trend towards hypocortisolemia was evident. However, we found positive correlations between corticosterone concentrations and anxiolytic responses, notably so with respect to the behavioural severity on day 7, but not on day 21. Earlier studies by Cohen *et al.* (2000) have found that lower levels of plasma corticosterone and prolactin in ketoconazole treated and PSE exposed rats were accompanied by lower anxiety levels compared to untreated exposed rats. This is essentially opposite to the findings described here, although the effects of chronic stress on corticosterone and ACTH secretion may vary depending on the experimental paradigm (Kant *et al.*, 1985; Cohen *et al.*, 2000). It has been reported that an adaptation to chronic stress may occur, which results in decreased plasma ACTH and corticosterone levels compared with levels following a single acute stressor which evokes an increase in glucocorticoids (Kant *et al.*, 1985; Kant *et al.*, 1987; Kanter *et al.*, 2001). It may therefore be possible that the current CORT data on day 21 reflects that of an acute stressor and where exaggerated negative feedback on the HPA-axis has not yet developed. This is typical of PTSD. Since hypercortisolemia is elicited to help deal with an aversive/stressful

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condition, stress resilient individuals may benefit from such an endocrine response by having a lower level of anxiety, indicative of better coping strategies under stress. On the other hand, an at-risk individual would respond in a maladaptive manner, presenting with the typical manifestations of anxiety. In fact, cortisol is responsible for containing the catecholamine system during stress; it assists in lowering the high concentrations of adrenaline that are released during 'fight or flight' (Yehuda, 2009). The worse scenario would be progression to increased HPA-axis suppression with hypocortisolemia, which may also be associated with anxiety, as has been demonstrated using the stress-restress model of PTSD (Harvey *et al.*, 2006). Therefore, in our hands the PSE model demonstrates robust face validity for PTSD, as demonstrated by the evidence of anxiety-like behaviour as noted in the EPM, hypocortisolemia, as well as the positive correlations between elevated plasma corticosterone and anxiolytic behaviour. Results in this model implicate a mutual interaction between corticosterone and NA in the pathogenesis of chronic anxiety following acute psychological trauma. More specifically, the role of NA seems to involve the α_{2C} AR.

As a dysfunctional noradrenergic system plays a central role in the pathology of PTSD, the third objective of this study was to investigate the role of selective antagonism of the α_{2C} AR with the novel compound, ORM-10921, administered 1-hour post PSE or 8 days post PSE, on the manifestation of PTSD-related anxiety and HPA-axis abnormalities. Considering the less than adequate response of clinical PTSD to modulation of the noradrenergic and serotonergic systems, i.e. chronic treatment with *inter alia* TCAs, SNRIs, and SSRIs, as well as doubts in the prophylactic activity provided by propranolol (Giustino *et al.*, 2016), there is a great need to identify new biological targets and develop more effective treatments for PTSD. The current investigation found that administration of a 0.3 mg/kg for 14 days beginning 7 days after PSE (Addendum C) induced significantly more anxiety like-behaviour in PSE rats. However, chronic administration of ORM-10921 (0.3 mg/kg x 21 days), starting immediately after PSE (Chapter 3), significantly reduced PSE-mediated behavioural disruptions. Therefore, an anxiolytic profile is evident in the behaviour of ORM-10921 treated animals. ORM-10921 also reversed a reduction in risk assessment and exploratory behaviour in PSE animals compared to saline treated PSE animals. These data not only confirm the efficacy of ORM-10921 to reverse PTSD-like aversive behaviour but suggest that the α_{2C} AR is causally linked to the genesis of anxiety in these animals, and by implication in PTSD.

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The time frame in which treatment was administered (1 hour after stress exposure) conforms to the time frame within which the memory consolidation process takes place at the cellular level. The time at which the effect was assessed was sufficiently distant from the stimulus as to suggest that the effect may well be mediated by memory related processes. The same pattern was observed in previous study in which anisomycin, a protein synthesis inhibitor, was effective in attenuating behavioural responses when administered within 1 hour after PSE exposure but not when administered later (Cohen *et al.*, 2006). This data suggests that ORM-10921 might have disrupted consolidation of short term fear memory to long-term memory, and as such providing an anxiolytic action.

Considering the primary action of ORM-10921 as a α_{2C} AR antagonist, we may postulate how such an action may evoke the above-described behavioural and neuroendocrine effects. α_{2C} ARs are expressed in a high density in the stress-regulatory regions of the brain, particularly in the amygdala, hippocampus and cortex (Berridge & Waterhouse, 2003). In the immediate post-PSE study (Chapter 3), inhibition of the α_{2C} AR would have promoted the release of NA (Sallinen *et al* 2007). However, although excessive NA is often associated with anxiety (Sallinen *et al* 2007), increased NA has also been linked to an anxiolytic action, probably via the action of NA on neighbouring serotonergic, GABAergic and glutamatergic neurons (Harvey & Slabbert, 2014). Research has demonstrated that drugs that alter glutamate transmission have potential anxiolytic action for many different paradigms including fear-potentiated startle, punished responding, and the elevated plus maze (Sallinen *et al.*, 2013). Human clinical drug trials have demonstrated the efficacy of anti-glutamatergic drugs for the treatment of obsessive-compulsive disorder, posttraumatic stress disorder, generalized anxiety disorder, and social phobia. Further, studies have shown that in the brain, α_{2A} - and α_{2C} -receptors inhibit dopamine release in basal ganglia (Bücheler *et al.*, 2002) as well as serotonin secretion in mouse hippocampus and brain cortex (Scheibner *et al.*, 2001).

To conclude, this study has provided valuable new information on the role of ORM-10921 in PTSD, demonstrating significant anxiolytic effects and showing therapeutic potential in preventing the development of acute stress syndrome into full-blown PTSD. The data have been obtained using a PSE model, thus increasing the overall validity and reliability of the findings. Moreover, these findings have contributed significantly to current knowledge regarding the neurobiology underlying PTSD and anxiety like behaviour, and how these

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processes can be pharmacologically targeted to alter the course of PTSD. The study has not only emphasized the central role of NA in PTSD, in particular the α_{2C} AR.

The study has established the predictive validity of the PSE model in our laboratory where it can now be applied in future exploratory studies into stress and anxiety disorders.

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

- A single 10-minute exposure to cat scent caused increased levels of anxiety behaviour in rats when tested 7 days later compared to the control non-exposed animals (Chapter 3).
- A single 10-minute exposure to cat scent induced hypocortisolemia when tested 21 days later, compared to the control non-exposed animals (Chapter 3).
- ORM-10921 (0.3 mg/kg) administered 8 days after exposure for 14 days (Addendum C) effectively increased bio-behavioural disruptions typical of PTSD, including subtle evidence for hypercortisolemia and anxiogenesis.
- Administration of ORM-10921 (0.3 mg/kg) immediately after PSE for 21 days (Chapter 3) reduced behavioural disruptions that are typical of PTSD, in particular reducing anxiety-like manifestations as well as a tendency to increase plasma corticosterone.

Shortcomings and future recommendations

- That the numbers of animals per group were not the same could possibly have prevented statistical significance in certain key analyses. However, this was unavoidable due to ethical constraints, the time frame of the study, and number of animals available. This can be avoided in future studies by ensuring enough time to breed sufficient animals and to have the same number of animals per group.
- Although it may be possible that uniform exposure to predator scent could have been compromised by the method used, we can confirm that cloth was exposed to the same male cat and only collected after a period of 2 months. Hereafter, it was sealed in a closed, air-tight bag. Further, gloves were used at all times in the handling of the cat and clean cloth to prevent any odour contamination. However, it was not possible to determine whether each 10 x10 cm cloth exuded the same intensity of smell. A possible way in which to address this in future would be to use laboratory scent that

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can be controlled. Important, the rats' olfactory system detects different volatile odour molecules, while the VNO specifically is specialized to receive within species messages (pheromones) relating to reproduction, aggression and defence (Halpern & Martinez-Marcos, 2003). Thus, it is improbable that concentration differences in scent would otherwise confound the current data.

- The fact that a dose response was not conducted in this study could possibly be a limitation as the novel compound ORM-10921 has never been tested in a PTSD model before. However, since a previous study in a rodent model of depression (Uys *et al.*, 2017) using ORM-10921 at a dose of 0.3 and 0.03 mg/kg showed pronounced antidepressant-like effects at a dose of 0.3 mg/kg, we are confident that this dosage used in this study is adequate. However, a 3-tiered dosage study would be beneficial in PTSD due to its complex mechanisms and neurobiology as well as comorbidities.
- Another limitation could possibly be that we did not know whether treatment response with ORM-10921 would be dependent on the time frame at which it is administered, either initiated immediately or distal to the traumatic event. However, we were able to address this potential drawback by undertaking the work presented in Addendum C, which demonstrated that delayed ORM-10921 treatment one week after trauma is less effective than immediately post-trauma administration (Chapter 3) and may even worsen the symptoms of PTSD.
- We further did not employ a positive control in the present investigation. While saline was employed as an appropriate negative control that forms the foundation of the current study, we originally intended venlafaxine as a positive control. Due to time restrictions and the less than foreseen availability of animals, this was unfortunately not possible in the end.
- The duration of treatment was another shortcoming in the study as we did not have a group of animals that were treated with ORM-10921 distal to the traumatic event for 7 days and another group for 21 days. This discrepancy could lead to difficulties in comparing data presented in chapter 3 to that described in Addendum C.
- Last, the natural progression of stress-related changes were not considered here since we did not have a group of animals only exposed to PSE (i.e. not treated) and assessed in the EPM on day 0, 7 and 21. However this was not part of the study as we aimed 1) to validate a useful animal model of PTSD, which we have indeed achieved as shown

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in the validation with groups I and II, and 2) to determine the response of immediate interference with either ORM or saline in this model.

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ADDENDUM A

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Guide for Authors

ENP

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17 November 2017

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PERMISSION TO SUBMIT WORK FOR EXAMINATION

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Yours sincerely

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De Wet Wolmarans
Study Co-Supervisor



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The article is as follows:

Manuscript A

The α_{2C} selective antagonist, ORM-10921, displays anxiolytic effects in an animal model of posttraumatic stress disorder

Sincerely,

A handwritten signature in black ink, appearing to read 'Brian H Harvey'.

Brian H Harvey, PhD
Study leader



Department of Psychiatry and Mental Health

Professor **Dan J Stein, FRCP, PhD, DPhil**

Head; Department of Psychiatry and Mental Health, UCT & GSH

Email: dan.stein@uct.ac.za

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The article is as follows:

Manuscript A

The α_2C selective antagonist, ORM-10921, displays anxiolytic effects in an animal model of posttraumatic stress disorder

Sincerely,

Dan J Stein

Prof Dan Stein

Co-author

ADDENDUM C

The effect of chronic treatment with ORM-10921, a selective α_{2C} adrenoceptor antagonist, from day 7 post predator scent exposure on neuroendocrine and behavioural responses

Introduction

As explained in Chapter 1 and 4, this study comprised of two treatment groups, namely (i) immediate post stressor treatment with ORM-10921 and (ii) delayed (8 days post-stressor) treatment with ORM-10921. There exists controversy regarding the most appropriate time to initiate pharmacotherapy after a severe traumatic event (Nemeroff *et al.*, 2006; Stein *et al.*, 2006). Re-experiencing, flash-backs and contextual reminders of the trauma prompts the progression of acute stress disorder (ASD) in the immediate aftermath of the trauma, to full-blown PTSD (Nemeroff *et al.*, 2006). This has prompted the use of propranolol, a non-selective $\beta_{1/2}$ AR antagonist, immediately post-trauma as a preventative strategy to curb the neurodevelopment of PTSD (Friedman, 1997). Its primary action is to inhibit noradrenergic-mediated consolidation of trauma and associated contextual memory in the amygdala and hippocampus (Vaiva *et al.*, 2003). In the main study (Chapter 3, Manuscript A), ORM-10921 was dosed immediately post-trauma for 21 days, in a manner congruent with how propranolol is administered clinically (Vaiva *et al.*, 2003). Nevertheless, in said chapter we highlight that a possible limitation to the study is that delayed ORM-10921 treatment from one week following trauma may have offered a better therapeutic response than acute post-trauma administration. To address this possibility, we undertook a similar study, but where ORM-10921 was administered subcutaneously over a treatment period of 14 days, beginning 8 days after PSE with behavioural assessments performed on day 7 and 21 and corticosterone analysis on 22. This allowed us to assess whether successful treatment with ORM-10921 is indeed dependent on whether treatment is initiated immediately or distal to the traumatic event. Unfortunately, these two studies could not be combined in Chapter 3 (Manuscript A) due to the different treatment durations used, viz. 14 vs. 21 days, for the delayed and immediate treatment cohorts respectively. The reason for this difference in treatment duration is that, while both 14 and 21 days are seen as chronic treatment, this enabled us to measure behaviour and endocrine responses on the exact same days for both treatment cohorts.

Methods

To assess the effects of ORM-10921 on the behaviour of rats one week following post-trauma, 100 male Wistar rats were exposed individually (while being video recorded) under dim white light (15 lux) for 10 min to either a 10 x 10 cm non-scented ($n = 31$) cloth, or a cloth exposed to a male cat (cat cloth) ($n = 69$). The groups therefore comprised non-exposed vehicle and

ORM-10921 (0.3 mg/kg/day) treated groups ($n = 12$ and 19 , respectively), as well as vehicle and ORM-10921 treated cat scent exposed groups $n = 37$ and 32 , respectively) in each case with treatment initiated on day 8 following exposure and continuing for 14 days.

As in Chapter 3, each rat was subjected to the EPM 7 days after exposure to measure anxiety-like manifestations and determine the percentage of well-adapted or maladapted cohorts. After 14 days of treatment a new set of behavioural tests in the EPM were carried out on day 21, with the rats sacrificed on day 22, trunk blood collected and stored until the day of corticosterone analysis (Figure 1). These results are presented on the following page.

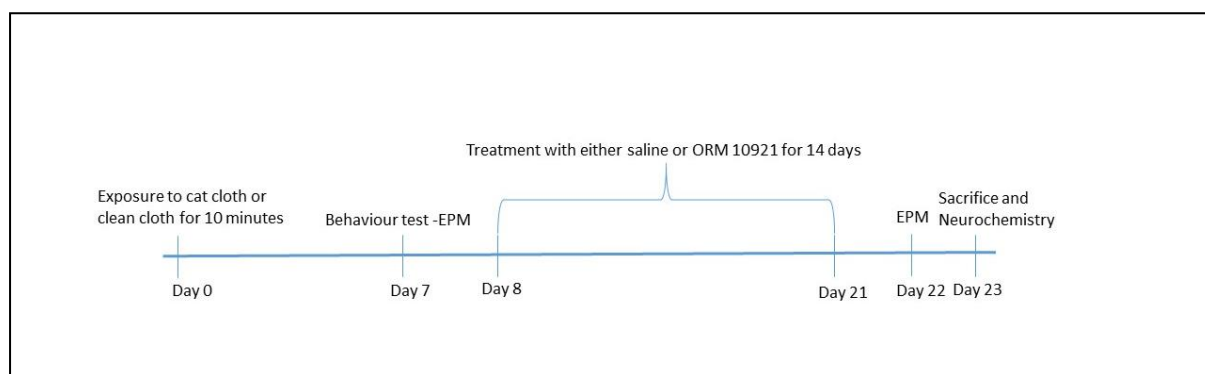


Figure 1 – Time line of delayed treatment in predator scent exposure model

Results

Open arm entries (Figure 2)

A student t-test with Bonferroni correction was conducted to compare non-exposed vs. exposed data on day 7 before the initiation of treatment, showing that PSE induced a significant decrease in open arm entries vs non-PSE controls (12.9 ± 0.91 vs. 9.8 ± 0.6 , $p = 0.006$), indicative of a significant anxiogenic effect. Data are presented as mean $\pm$ standard error of the mean (SEM), unless otherwise stated.

Following two-way repeated measures ANOVA [$F(1,67) = 0.004$, $p = 0.95$], pairwise comparisons were run for day 7 vs. day 21 for both saline and ORM-10921-treated cohorts, with a reported 95% confidence interval and Bonferroni adjusted p -values within each simple main effect. Saline exposed animals on day 21 had a statistically significant reduction in the number of open arm entries vs day 7 (8.70 ± 1.03 vs. 11.04 ± 1.00 ; $p = 0.05$), indicative of greater anxiety evident on day 21 vs. day 7 post-PSE. Further, this reduction was not reversed by 14-day ORM-10921 treatment.

Closed arm entries (Figure 3)

A student t-test was conducted to compare non-exposed vs. exposed day 7 pre-treatment data, showing that PSE failed to evoke a significant difference in closed arm entries in saline-treated PSE animals vs. their non-PSE controls (12.8 ± 0.71 vs. 14.1 ± 0.97 , $p = 0.4$).

Following two-way repeated measures ANOVA [$F(1,67) = 1.942$, $p = 0.16$] pairwise comparisons were run for day 7 vs. day 21 for both the saline and ORM-10921-treated cohorts, with a reported 95% confidence interval with the p -values Bonferroni adjusted within each simple main effect. ORM exposed animals had a statistically significant reduction in the number of closed arm entries on day 21 compared to saline exposed day 21 (10.7 ± 1.1 vs 15.4 ± 1.1 ; $p = 0.01$), indicative of a significant anxiolytic-like action with respect to closedarm entries of ORM-10921 in PSE animals as assessed on day 21.

3.1.1 Time in open arms (Figure 4)

A student t-test was conducted to compare non-exposed vs. exposed pre-treatment data on day 7, showing that saline treated PSE induced a significantly decreased time in the open arm vs non-PSE controls (115.7 ± 10.2 vs. 86.1 ± 7.95 s, $p = 0.03$), indicative of anxiety prevalent in these animals. Data are presented as mean $\pm$ standard error of the mean (SEM), unless otherwise stated.

Following two-way repeated measures ANOVA [$F(1,67) = 0.3369$, $p = 0.56$] pairwise comparisons were run for day 7 vs. day 21 of both the saline and ORM-10921 treated cohorts. Saline treated animals presented with a statistically significant reduction in the time spent in open arm on day 21 vs day 7 (52.4 ± 8.1 vs 81.4 ± 9.8 ; $p = 0.05$), indicative that anxiety worsened between days 7 and 21 in saline-treated PSE animals. However, ORM-10921 treated animals also presented with a statistically significant reduction in the time spent in open arms on day 21 compared to day 7 (52.6 ± 8.8 vs. 91.4 ± 12.9 ; $p = 0.01$), suggesting it not only failed to reverse anxiety-like behaviour in PSE rats, instead sustaining it. This finding is in direct contrast to the results presented in Chapter 3.

3.1.2 Time spent in closed arms (Figure 5)

A student t-test was conducted to compare the time spent in the closed arms by non-exposed vs. exposed animals on day 7 before initiation of treatment, demonstrating that PSE rats spent significantly more time in the closed arms vs. non-PSE rats (147.4 ± 0.6 vs. 115.2 ± 7.6 s, $p = 0.01$). This parameter was also not worsened when compared to time in closed arms on day 21. Finally, no statistically significant difference was found for saline or ORM-10921 treated cohorts with regards to time spent in closed arms, with a trend towards more time spent in the closed arm, thus indicating a subtle anxiogenic response on the part of ORM-10921.

3.1.3 Number of head dipping episodes (Figure 6)

A student t-test was conducted to compare the number of head dipping episodes made by the non-exposed vs. exposed animals on day 7 pre-treatment, showing that PSE rats had significantly reduced head dipping episodes vs. their saline controls (17.6 ± 2.2 vs. 12.5 ± 1.1 , $p = 0.02$), indicative of anxiety. No statistically significant was found for saline or ORM-10921 treated cohorts with regards to number of head dipping episodes.

3.1.4 Plasma corticosterone (Figure 7)

Two-way ANOVA was conducted to examine the effects of drug and exposure on the concentration of corticosterone in blood plasma on day 22. The interaction effect between drug and exposure was not statistically significant [$F(1, 78) = 2.403, p = 0.1252$]. Therefore, an analysis of the main effects for both drug and exposure was performed, which indicated that the main effect of exposure was statistically significant [$F(1, 78) = 9.012, p = 0.0036$].

Bonferroni multiple comparison tests failed to note any difference between saline non-exposed vs. exposed animals, indicating that PSE did not alter plasma corticosterone, while corticosterone levels also did not change between days 7 and 21. However, a statistically significant increase in corticosterone was observed in the ORM exposed animals vs. ORM non-exposed animals (70.54 ± 13.28 vs 192.3 ± 33.71 ; $p \leq 0.01$), implying that ORM-10921 prompted an elevation in corticosterone.

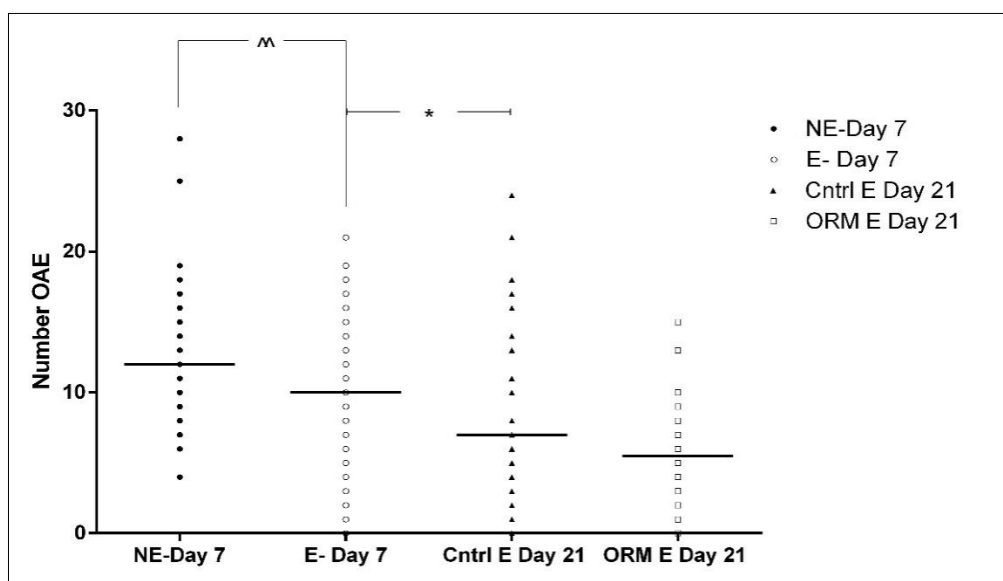


Figure 2 – Number of open arm entries of exposed and non-exposed saline treated group compared to exposed and non-exposed ORM-10921 treated groups. Student t-test ^^ NE vs E day 7 (12.9 ± 0.9 vs. 9.8 ± 0.62 , $p = 0.006$); pairwise comparison on day 21 vs day 7 * 8.7 ± 1.0 vs. 11.0 ± 1.0 ; $p \leq 0.05$. NE = non-exposed, E = exposed and Cntrl = saline treated

ADDENDUM C

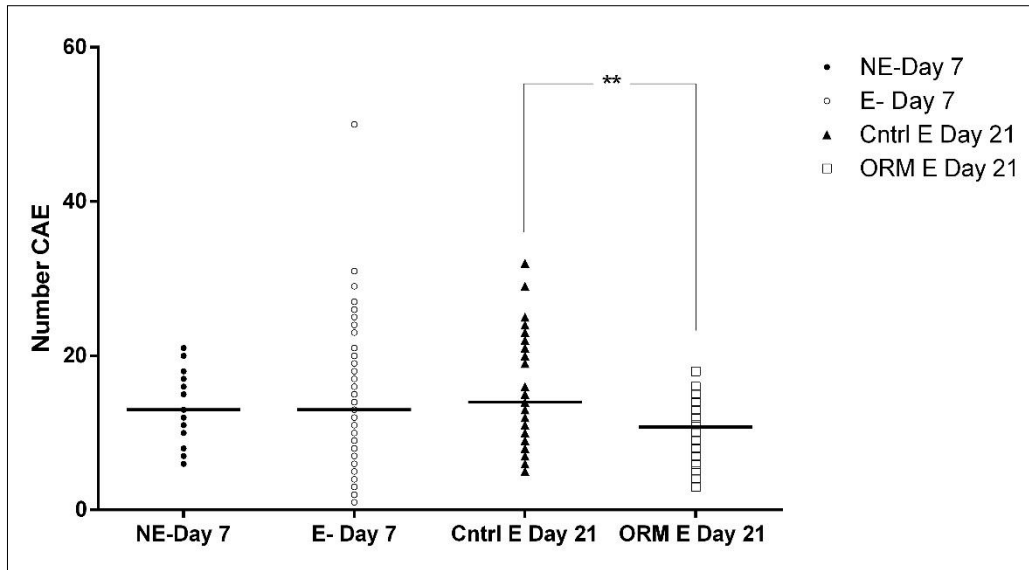


Figure 3 – Number of closed arm entries made by the exposed and non-exposed saline treated groups compared to exposed and non-exposed ORM-10921-treated groups. Pairwise comparison on day 21 10.7 ± 1.0 vs. 15.4 ± 1.07 ; $p \leq 0.01$ NE = non-exposed, E = exposed and Cntrl = saline treated

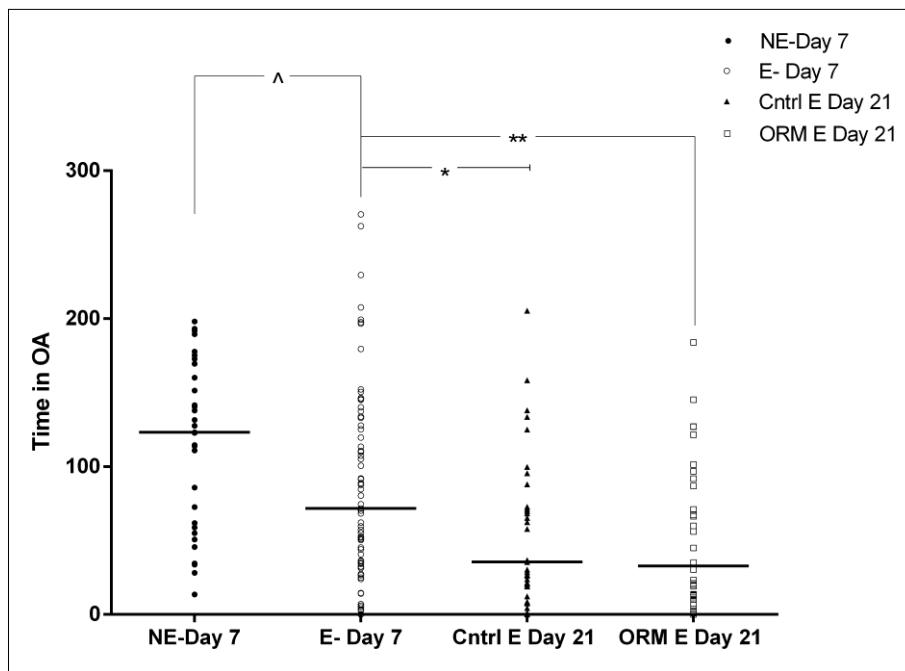


Figure 4 – Time spent in the open arms by the exposed and non-exposed saline treated groups compared to exposed and non-exposed ORM-10921-treated groups. Student t-test ^NE vs E day 7 (115.7 ± 10.2 vs. 86.1 ± 7.9 s; $p = 0.03$) and pairwise comparison between saline exposed day 21 vs. 7 52.4 ± 8.1 vs. 81.4 ± 9.8 s; $p \leq 0.05$) and ORM-10921 day 21 vs. day 7 52.6 ± 8.8 vs. 91.4 ± 12.9 s; $p \leq 0.01$). NE= non-exposed, E=exposed and Cntrl= saline treated

ADDENDUM C

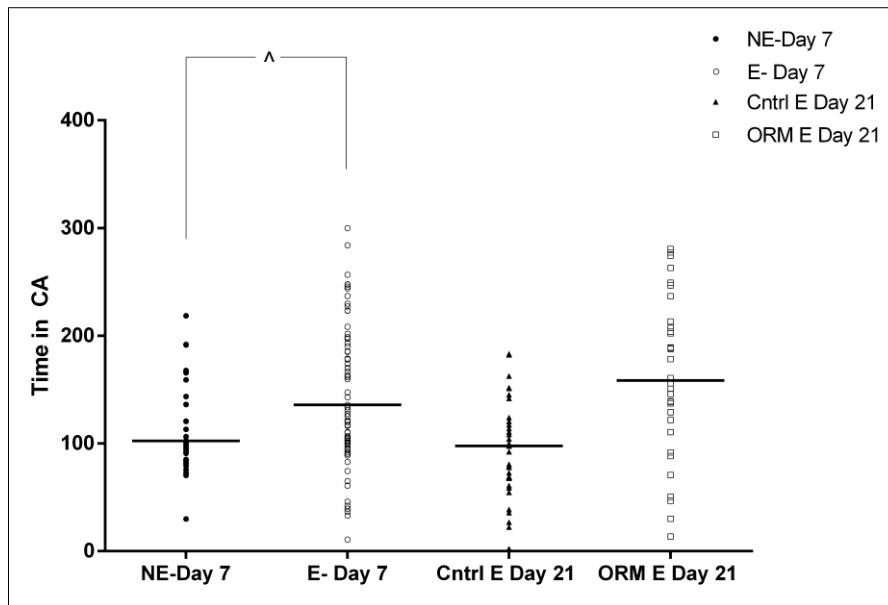


Figure 5 – Time spent in the closed arms of exposed and non-exposed saline treated groups compared to exposed and non-exposed ORM-10921-treated groups. Student t-test ^^ NE vs E day 7 (115.2 ± 7.6 vs. 147.4 ± 0.6 s, $p = 0.01$). NE= non-exposed, E=exposed and Cntrl= saline treated

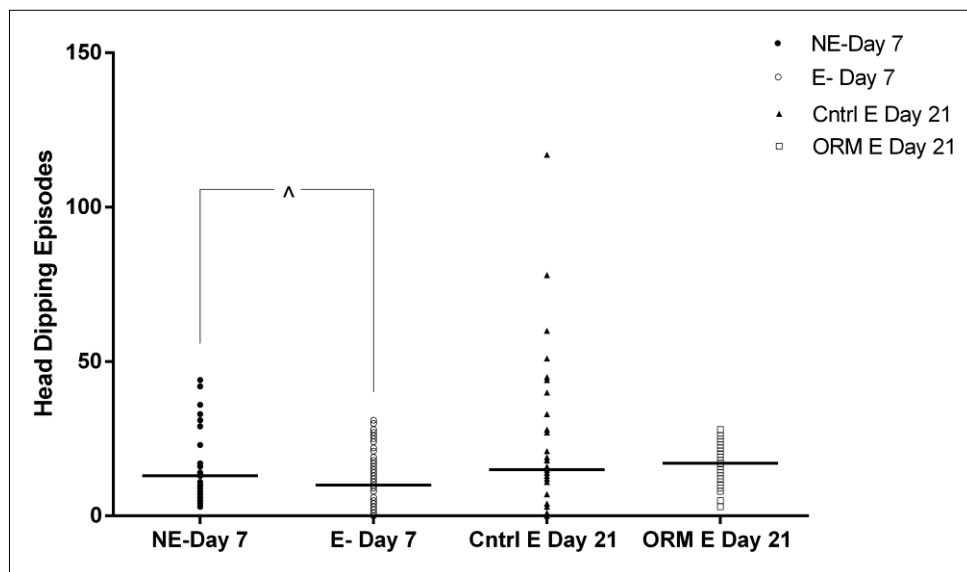


Figure 6 – Number of head dipping episodes of exposed and non-exposed saline treated groups compared to exposed and non-exposed ORM-10921-treated groups. Student t-test ^ NE vs E day 7 (17.6 ± 2.3 vs. 12.5 ± 1.1 , $p = 0.02$) NE= non-exposed, E=exposed and Cntrl= saline treated

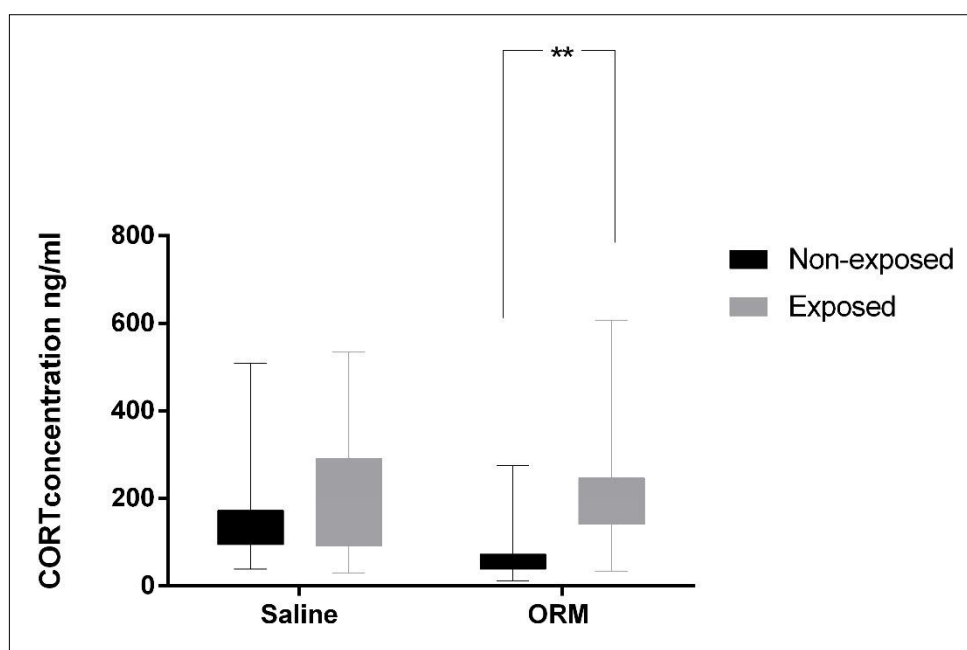


Figure 7 – Concentration of Corticosterone in the exposed and non-exposed saline treated groups and exposed and non-exposed ORM-10921-treated groups. (70.54 ± 13.28 vs 192.3 ± 33.71 ; $p \leq 0.01$).

Conclusion

In conclusion the above data suggests that PSE is anxiogenic when assessed on day 7 post-exposure, as shown in the EPM data (Figures 2 – 6), with evidence that said anxiety worsens between days 7 and 21 in the absence of treatment (Figures 2 and 4). ORM-10921 administered from day 8 post PSE, demonstrated confounding effects regarding measures of anxiety on day 21, being anxiolytic with respect to closed arm entries and anxiogenic when assessed using other parameters, such as time in open arms (Figure 4) and time spent in closed arms (Figure 5). Considering plasma corticosterone levels, despite PSE inducing anxiety, it failed to alter plasma corticosterone in any meaningful way (Figure 7), while this response also did not change over time (comparing day 7 to day 21). Interestingly, ORM-10921 tended to elevate corticosterone levels.

That ORM-10921 failed to reverse PSE-induced anxiety in this study, could be due to the time frame at which the drug was administered, as it is possible that delayed treatment might not be able to block the excessive consolidation of fear memories and/or fear conditioning associated with the anxiety like behaviour over time. In fact, anxiety worsens over time (Figure 4). However, it also emphasizes the shortcomings of this part of the study, since we only treated for 14 days and not 21 days; it could be possible that an extra 7 days of treatment could have presented with more ameliorating therapeutic effects. However, the study

confirms that the protocol applied in Chapter 3 is indeed the most appropriate regimen and that ORM is more effective as a prophylactic agent to prevent the neurodevelopment of PTSD than it is when administered some time distal to the trauma. Thus, this data lays an important foundation for future studies with regard to time of treatment as well as duration of treatment.

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The End
