

Efficacy and Mechanism of Action of Intrahippocampal D-cycloserine in an Animal Model of Posttraumatic Stress Disorder (PTSD)

by

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DECLARATION

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Abstract

Posttraumatic stress disorder (PTSD) has been described as a persistent (Bremner et al., 1996) and incapacitating (Zatzick et al., 1997; Mendlowicz & Stein 2000) psychiatric disorder which occurs after exposure to a potentially traumatic event (DSM-5, APA 2013). Exposure based therapy (EBT) is one of the most common and effective therapies for posttraumatic stress disorder (Mendes et al., 2008). The procedure involves controlled exposure to the feared stimulus in the absence of any overt danger. EBT in humans is procedurally very similar to fear extinction training in animal models of emotional learning, such as fear conditioning (Norton & Price, 2007). It has previously been shown that dysfunctional fear extinction underpins PTSD pathophysiology (Keane et al., 1985; Cohen et al., 2006; Amstadter et al., 2009). With recent studies demonstrating fear extinction as an essential process for studying putative pharmacotherapies for clinical use in PTSD treatment. One such novel pharmacological agent, D-cycloserine (DCS), has been investigated in both preclinical and clinical studies of anxiety and has been reported to facilitate extinction of learned fear in rats and to promote exposure-based therapies in humans (Walker et al., 2002; Ledgerwood et al., 2003; Davis et al., 2006; Bontempo et al., 2012).

DCS is a partial agonist of the N-methyl-D-aspartate receptor (NMDAR) and exerts its effects by binding to the glycine regulatory site of the NMDA complex. In addition, these glutamatergic receptors, specifically the hippocampal NMDARs and their subsequent signalling pathways have been implicated in fear extinction. PTSD affects individuals in all sectors of society and is as much a concern with respect to children as to adults. Considering that adolescence is a period of heightened vulnerability for mood and anxiety disorders, it is crucial to observe the effects of trauma on this developmental stage.

Therefore the main aim of this study was to determine whether intrahippocampal infusions of DCS could reverse the PTSD phenotype, as displayed by our animal model, in both adolescents and adults with special focus on fear extinction. In the current study, we used a fear conditioning paradigm consisting of a brief, intense electric footshock (1.5 mA) and a neutral tone (80 dB, 9 kHz) to represent the traumatic event and investigated the efficacy and molecular mechanism of action of DCS on the behaviour and neurochemistry of adolescent and adult rats. The present study was particularly interested in the effects of DCS on hippocampal brain-derived neurotrophic factor (BDNF), hippocampal NMDAR expression levels, factors downstream of the NMDA signalling pathway (i.e. neuronal nitric oxide synthase) and protein changes in the hippocampus (HC) of fear conditioned and DCS-treated animals.

Our animal model generated the following key findings. Firstly, various behavioural tests demonstrated that fear conditioned rats exhibited a PTSD-like disorder as shown by their increased and sustained conditioned fear response and increased anxiety-like symptoms. These effects were reversed by intrahippocampal DCS infusions, as assessed by behavioural freezing. Secondly, an upregulation of hippocampal NMDARs was noted in fear conditioned rats, while repeated administration of intrahippocampal DCS reduced this effect. Thirdly, intrahippocampal DCS infusions enhanced dorsal hippocampal BDNF expression in DCS treated groups, with fear conditioned rats expressing the lowest BDNF levels. Fourthly, intrahippocampal DCS administration elicited similar patterns in adolescents and adults with regards to fear extinction i.e. a decreased fear response was noted in both age groups after DCS administration. Lastly, we observed that hippocampal protein expression differed between adolescent and adult rats. Most proteins were distinctly expressed in either of the two age groups. The protein, neurabin-2 was specifically expressed during the adolescent period. Furthermore, footshock led to an increase in adolescent protein expression, whereas DCS treatment led to a decrease in adolescent protein expression.

Overall, this study supported and extended previous findings that DCS has a therapeutic effect on fear conditioning by enhancing extinction of anxiety-like symptoms in rodents. We were able to show that animals subjected to fear conditioning/trauma show signs of alterations in proteins involved in neuronal plasticity, calcium (Ca^{2+}) homeostasis, cellular stress, cell cycle arrest, initiation of apoptotic mechanisms and cell signalling dysregulation. These proteins all have a role in one or more of the neurochemical parameters as examined in our PTSD model i.e. interact with the HC, BDNF, nNOS or NMDARs. Therefore, additional studies are needed to elucidate the relationship between epigenetic modifications and the resulting proteomic responses as demonstrated in our study. In addition, the role of BDNF in PTSD has to be further investigated, be it as a biomarker or as adjunctive therapy for PTSD.

“The aim of treating PTSD is to enable patients to live in the present with freedom from feelings or behaviors that belong in the past.” - David J. Nutt

Opsomming

Posttraumatisiese stresversteuring (PTSV) is 'n ernstige en uitmergelende psigiatriese versteuring, wat na blootstelling aan 'n potensieel traumatiese gebeurtenis, kan voorkom (DSM-5, APA 2013). Dit blyk dat terapie wat gebaseer is op blootstelling (blootstelling gebaseerde terapie) die mees algemene en effektiewe terapie vir PTSV is (Mendes et al., 2008). Die procedure behels blootstelling aan 'n stimulus wat vrees inboesem en word ondergekontroleerde of beheerde toestande uitgevoer, dus in die afwesigheid van direkte gevaar. Die prosedure in die behandeling van mens toon sterk ooreenkomste met die van diere modelle met spesifieke verwysing na vrees kondisionering waar die klem op emosionele onderrig en vrees uitwissing val (Norton en Price, 2007). Voorheen is bewys dat PTSV op 'n patofisiologiese wyse onderliggend is aan disfunksionele vrees uitwissing (Keane et al, 1985, Cohen et al, 2006, Amstadler et al, 2009). Onlangse studies toon die belangrikheid en fenomenale impak van vrees uitwissing as 'n noodsaaklike proses het op huidige en toekomstige studies vir farmakologiese terapie en kliniese behandeling van PTSV. So byvoorbeeld is die farmakologiese middel, D-cycloserine (DCS) getoets in prekliniese en kliniese studies waar aanvanklike ondersoek is. Daar is bevind dat DCS die uitwissing van aangeleerde angste in rottefasiliteer en dit .bemarkook blootstelling gebaseerde terapie wat vir die mens van belang is (Walker et al 2002, Ledgerwood et al 2003, Davis et al 2006, Bontempo et al 2012)

DCS is 'n gedeeltelike agonis van die N-metiel-D-aspartaat reseptor (NMDAR) en beoefen sy uitwerking deur binding met die glisien regulatoriese plek van die NMDA kompleks. Bykomend by vrees uitwissing word dié glutamaat reseptore, met spesifieke verwysing na die hipocampus (HC) NMDARs en hul gevolglike sein paaie betrek. PTSV affekteer individue van alle vlakke van die samelewing en is 'n groot kommer wat kinders sowel as volwassenes betref. Gegee die feit dat adollessensie 'n tydperk van verhoogde/toenemende weerloosheid ten opsigte van buie en angs versteurings is, is dit noodsaaklik om die uitwerking van trauma op dié ontwikkelings stadium waar te neem.

Derhalwe was die hoofdoel van hierdie studie om vas te stel of intrahippokampus infusies van DCS die teenoorgestelde van die PTSV fenotipe kan wees soos uitgebeeld deur die dieremodel in beide adollesente en volwassene stadia met spesifieke fokus op vreesversteurings. In die huidige studie het ons 'n vrees gekondisioneerde paradigma bestaande uit 'n kort intense elektriese skok (1,5 mA) en 'n neutrale toon (80 dB, 9kHz) gebruik om die traumatiese gebeurtenis te verteenwoordig en die doeltreffendheid en molekulêre meganisme van DCS op die gedrag en neurochemie van die adollesent en volwasse rotte te ondersoek. Ons studie was veral gemik op die uitwerking van DCS op die HC, die brein-afkomstige neurotrofiese faktor, hippokampus NMDAR uitdrukingsvlakke, faktore onderliggend aan die NMDA sein paaie (nNOS) en proteïenveranderinge in die HC van vrees gekondisioneerde en DCS behandelde diere.

Die volgende bevindinge is gemaak op grond van ons dieremodel: Eerstens het verskeie gedragstoetse gedemonstreer dat angs gekondisioneerde rotte 'n PTSV gewyse versteuring openbaar. Dit is bewys deur hul toenemende en volgehoue gekondisioneerde vrees respons en toenemende angs-gewyse simptome. Hierdie effekte is weerspreek deur die intrahippokampus infusies van DCS, soos geassesseer deur die vreesgedrag van die diere. Tweedens is 'n opgradering van HC NMDAR in vrees gekondisioneerde rotte waargeneem, terwyl herhaalde toediening van intrahippokampus DCS hierdie effek verlaag het. Derdens verbeter intrahippokampus DCS infusies die dorsal hippokampus BDNF uitdrukking in DCS behandelde groepe, met vrees gekondisioneerde rotte wat die laagste BDNF vlakke toon. Vierdens het intrahippokampus DCS toediening wat vreesuitwissing betref, soortgelyke patrone in adollesente en volwassenes meegebring; dit is 'n afnemende vreesrespons wat in beide ouderdomsgroepe na DCS toediening waargeneem is. Laastens het ons waargeneem dat die HC proteïene uitdrukking tussen adollesente en volwasse rotte verskil. Die meeste proteïene was onderskeidelik uitgedruk in enigeen van die twee ouderdomsgroepe. 'n Unieke proteïen,

naamlik neurabin-2 was spesifiek uitgedruk gedurende die adollesente tydperk. Gevolglik het voetskok gelei tot 'n toename in adollesente proteïene uitdrukking, teenoor DCS behandeling wat 'n afname in adollesente proteïene uitdrukking tot gevolg gehad het.

In oorsig, ondersteun hierdie studie ons alternatiewe hipoteses en uitgebreide vroeëre bevindings dat DCS 'n terapeutiese uitwerking op vreeskondisionering het en DCS verbeter die uitwissing van angsgewyse simptome in knaagdiere. Ons was in staat om te bewys dat diere wat aan vreeskondisionering/trauma onderwerp was, tekens getoon het van verandering in proteïene betrokke in neuronale plastisiteit, kalsium homeostase, sellulêre stress, selsiklus arrestasie, aanvang van apoptotiese meganismes en sellulêre sein disregulasie. Hierdie proteïene het almal 'n rol gespeel in een of meer van die neurochemiese parameters so in ons PTSV model ondersoek is; genaamd die interaskie van die HC, BDNF, nNOS en NMDAR. Derhalwe is verdere addisionele studies nodig om die verwantskap tussen epigenetiese modifikasies en die gevolglike proteomiese response, soos in ons studie gedemonstreer, uit te brei. Dit is ook nodig dat die rol van BDNF in PTSV verder ondersoek moet word, hetsy as 'n bio-merker of as bykomende terapie vir PTSV.

“The aim of treating PTSD is to enable patients to live in the present with freedom from feelings or behaviors that belong in the past.” - David J. Nutt

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Abbreviations

α alpha

1st first

2nd second

5-HTT serotonin transporter

5-HTTPLPR serotonin transporter-linked polymorphic region

μ g micrograms

μ l microlitres

μ m micrometers

III three

IV four

IV-TR four text revised

V five

$^{\circ}$ C degrees Celsius

% percentage

AAALAC Association for Assessment and Accreditation of Laboratory Animal Care

ACN acetonitrile

ADP adenosine diphosphate

AHP afterhyperpolarization

A.M. ante meridium

AMPA α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA	analysis of variance
AP	anterior posterior
APA	American Psychological Association
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
Ca ⁺	calcium
CaM	calmodulin
CAMKII	calcium/calmodulin kinase II
cAMP	cyclic adenosine monophosphate
CBT	cognitive behavioural therapy
cGMP	cyclic guanosine monophosphate
C DCS	control D-cycloserine
CDK 5	cyclin-dependent kinase 5
cm	centimetres
CR	conditioned response
CS	conditioned stimulus
C Sal	control saline
Ctsc	cathepsin C
Cybb	Cytochrome b-245, beta polypeptide
Cyp7b1	oxysterol 7 α -hydroxylase
dB	decibel

DCS	D-cycloserine
DHC	dorsal hippocampus
DNA	deoxyribonuclear acid
DSM	Diagnostic and Statistical Manual
DV	dorsoventral
EBT	exposure-based therapy
ELISA	enzyme-linked immunosorbent assay
EMDR	eye movement desensitization and reprocessing
ERK	endoplasmic reticulum kinase
Fabp7	fatty acid binding protein 7
Fear DCS	fear conditioned D-cycloserine
Fear Sal	fear conditioned Saline
FK506	facrolimus (binding protein)
FKBP5	FK506 binding protein 5
fMRI	functional magnetic resolution imaging
FST	forced swim test
g	grams
GluR1	glutamate receptor subunit 1
GMP	guanosine monophosphate
GxE	gene environment interactions
h	hour

HC	hippocampus
HPA	hypothalamic pituitary axis
HPLC	high performamance liquid chromatography
HSP	heat shock protein
Htr2c	hydroxytryptamine receptor 2C
Hz	hertz
Il1rn	Interleukin 1 receptor antagonist
Inc.	Incorporated
IP3	inositol triphosphate
iTRAQ	isobaric Tagging for Relative and Absolute Quantitation
kDa	kilo Dalton
kg	kilograms
kHz	kilo Hertz
kV	kilo Volt
<i>LC</i>	<i>liquid chromatography</i>
LC-NA	locus coeruleus-noradrenergic
L/D	light/dark
LTD	long-term depression
LTM	long-term memory
LTP	long-term potentiation
M	molar

mA	milli Ampere
MALDI	matrix assisted laser absorption ionization
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
mBDNF	mature brain-derived neurotrophic factor
mg	milligrams
Mg ⁺	Magnesium
min	minutes
mm	millimetres
mM	millimolar
Mmp9	Matrix metalloproteinase 9
mRNA	messenger ribonucleic acid
MS/MS	tandem mass spectrometry
n	number (total)
N	naïve
NaCl	sodium chloride
NaClO ₄	sodium perchlorate
NAD	Nicotinic acid dihydrate
NADPH	nicotinamide adenine dinucleotide phosphate
Na ⁺ /K ⁺	sodium/potassium
NGF	nerve growth factor

NMDA	N-methyl-d-aspartate
NMDAR	N-methyl-d-aspartate receptor
NMDAR1	N-methyl-d-aspartate receptor subunit 1
NMDAR2	N-methyl-d-aspartate receptor subunit 2
nNos	neuronal nitric oxide synthase
NO	nitric oxide
NP-40	nonidet P-40
NR1	N-methyl-d-aspartate receptor subunit 1
NT-3	neurotrophic factor 3
pAKT	phosphorylated AKT
PARP	Poly ADP Ribose Polymerase
PBS	processing bodies
pCREB	phosphorylated cyclic AMP response element binding protein
pERK	pancreatic endoplasmic reticulum kinase
pg	picogram
pH	power of hydrogen
PI3K	phosphorinotide 3-kinase
PKA	Protein kinase A
PKG	Protein kinase G
P.M.	post meridium
pmol	picomol

PND	postnatal day
PP	ProteinPilot
PP-1	protein phosphatase-1
pTrk	phospho-tropomyosin related kinase
PTSD	posttraumatic stress disorder
RB	ribonuclear
ROS	reactive oxygen species
rpm	revolutions per minute
s	seconds
SEM	standard error of the mean
SPS	single prolonged stress
Tacr3	Tachykinin receptor 3
TPP	TransProteomic Pipeline
Trh	thyrotropin-releasing hormone
Trk	tropomyosin related kinase
TDS	time-dependent sensitization
VHC	ventral hippocampus
Vim	Vimentin
US	unconditioned stimulus
USA	United States of America

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Chapter 8:

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Chapter1

Introduction to the study

1.1 Introduction

Posttraumatic stress disorder (PTSD) manifests after exposure to a potentially traumatic event (DSM-5, APA 2013) and has been described as a persistent (Bremner et al., 1996) and incapacitating (Zatzick et al., 1997; Mendlowicz & Stein 2000) psychiatric disorder. PTSD symptoms include re-experiencing the trauma through intrusive flashbacks/nightmares, avoidance/emotional numbing, hyperarousal and negative changes in mood and cognition (DSM-5, APA 2013). Recent studies further suggest that dysfunctional fear extinction plays a fundamental role in the development of PTSD pathophysiology (Li et al., 2005; Adamec et al., 2006; Milad et al., 2009). Therefore, several PTSD therapies used for both children and adults are based on the processes of fear extinction (Norton & Price, 2007).

Earlier exposure based therapy (EBT) was considered the first-line treatment for PTSD, as its efficacy had been demonstrated in a large number of patients (Foa et al., 1991, 1993, 1995; Norton & Price, 2007). However, some patients still experienced frequent relapses; prompting considerable interest in developing more successful treatments for this stress- and trauma-related disorder. As extinction is procedurally very analogous to EBT in humans (Zarate & Agras, 1994), a number of pharmacological agents has been developed focusing on the underlying molecular processes of EBT. Subsequently a range of novel drugs has been studied in order to improve the management of PTSD (Goldstein et al., 2001; Wrubel et al., 2007; Bredy et al., 2007; Bredy & Barad, 2008; Quirk & Mueller, 2008; Chang et al., 2009; Graham & Richardson, 2009 b; Milad et al., 2009).

D-cycloserine (DCS) is a drug that was initially used in the treatment of tuberculosis (Mandell & Sande, 1990). Interestingly its potential therapeutic benefits were also suggested for an array of anxiety disorders (Ressler et al., 2004; Hofmann et al., 2006; Guastella et al., 2007; Kushner et al., 2007; Wilhelm et al., 2008; Otto et al., 2010 b). Furthermore, DCS appeared to successfully reduce the rate of relapse in a rodent model of PTSD (Richardson et al., 2004). Promising results such as these have led to DCS and its facilitative effects to be currently investigated in clinical studies of PTSD (Ponomarev et al., 2010; de Kleine et al., 2012; Litz et al., 2012; Ren et al., 2013). Preliminary data from these clinical studies support DCS augmentation of EBT, specifically in patients with more severe PTSD who required longer treatment. While the effectiveness of DCS to bring symptomatic relief is emerging, insights into how this drug facilitates this improvement remain limited.

A few studies have attempted to unravel the molecular mechanism of action of DCS. For example DCS has been found to bind to the glycine regulatory site of the *N*-methyl-*D*-aspartate receptor (NMDAR) complex (Sheinin et al., 2001) where it acts as a partial agonist. Activation of these glutamatergic receptors and their subsequent signalling pathways has been suggested to mediate long-lasting negative behavioural effects triggered by traumatic events (Sziray et al., 2006). In particular, the glutamatergic NMDAR system of the limbic brain structures have been implicated in the establishment of these unwanted behavioural consequences (Walker et al., 2002; Ledgerwood et al., 2005).

Since PTSD is considered an abnormality in fear extinction, clinical (Rauch et al., 2006; Sehlmeier et al., 2009) and animal (Sotres-Bayon et al., 2004; Bouton et al., 2006) studies have focused on the role of limbic structures in the neural circuitry of fear. Interactions between the amygdala, medial prefrontal cortex and hippocampus (HC) have been shown

to modulate fear extinction (Rauch et al., 1998; Quirk & Mueller, 2008). These structures are essential for the acquisition and consolidation of conditioned fear and extinction, processing of contextual information and retrieval of extinction learning (Kim & Jung, 2006; Shin & Liberzon, 2010; Kaplan et al., 2011). The function of the HC in PTSD has been extensively studied due to its central role in learning and memory (Mizomuri et al., 2007). Alterations in the HC have been proposed by many investigations to be primary to the development of mood and anxiety disorders such as PTSD (Duman et al., 1999; McEwen, 1999; Sotres-Bayon et al., 2004; Corcoran & Maren, 2004; Bouton, 2004; Bouton et al., 2006). The HC has specifically been found to play a vital role in fear extinction (Szapiro et al., 2003; Barad, 2005) and given its high expression levels of NMDARs, the present study also focused on this brain area.

Neurotrophins are growth factors that are essential for neuronal survival and differentiation. Previous studies have shown that adverse environments may affect hippocampal neurotrophin levels (Roceri et al., 2002; Uys et al., 2006; Faure et al., 2007). Given that reduced hippocampal volumes have been observed in PTSD (Woon et al., 2010) it is likely that neurotrophin levels may be decreased in these patients (Shirayama et al., 2002; Dell'Osso et al., 2009). The present study subsequently measured the levels of brain-derived neurotrophic factor in the HC as an indicator of changes in neurotrophin levels that may be associated with PTSD and DCS treatment. The present study also assessed neuronal nitric oxide synthase (nNOS) concentrations to determine the possible involvement of nitric oxide in PTSD and DCS-mediated effects.

1.2 Rationale for the study

Although the neural circuits underlying fear extinction have been identified, some of the molecular mechanisms involved remain to be elucidated. The proposed study has been

designed to fill gaps in our current knowledge especially in the context of the current limited availability of effective therapeutic strategies for fear extinction, and as such for PTSD.

The animal model of Siegmund et al. (2007) was chosen as a basis and modified for the purpose of this study. In essence a brief but intense electric footshock was applied and served as traumatic event. This approach prohibited the induction of habituation. This model also allowed for core behaviours (e.g. freezing) to be repeatedly measured and provided a platform to distinguish affected from non-affected animals.

The developmental stage of the subject at the time of exposure to the traumatic event is important for the evolution of PTSD. Specifically during adolescence, ontogenetic alterations occurring in brain function are vital in the development of stress-related psychopathologies (Bogerts, 1989; Compas et al., 1993; Lipska & Weinberger, 1993; Petersen et al., 1993, 1996; Spear, 2000). For instance studies have demonstrated that adolescents subjected to traumatic events have enhanced stress responses which often manifests as hypervigilance (Allen & Matthews, 1997; Spear, 2000). Exposure to stress during this critical developmental period has therefore been suggested to underpin the later development of stress-related psychopathologies (Spear, 2000, 2004; Heim & Nemeroff, 2001; Heim et al., 2004; Maercker et al., 2004; Nemeroff, 2004; Pynoos et al., 1999; McGivern et al., 1996; Einon & Morgan, 1977). The present study subsequently compared the effects of brief electric footshocks administered to adolescents to that observed in adults.

As mentioned earlier neurotrophins maintain neuronal survival, growth, differentiation, synaptic plasticity and efficacy (Lo, 1996). Previous experiments in our laboratory demonstrated decreased neurotrophin levels such as brain-derived neurotrophic factor (BDNF) in response to a traumatic event (Uys et al., 2006; Faure et al., 2007). Given the functional properties of neurotrophins, it is reasonable to hypothesize that altered BDNF levels, accompanying a traumatic event, may constitute a compensatory mechanism. This hypothesis merits further investigation into whether manipulation of BDNF concentrations may enhance fear extinction.

Traditional exposure based therapy involves protein synthesis dependent consolidation-like mechanisms. Enzyme-linked immunosorbent assays (ELISAs) and Western Blot techniques represents one way in which these proteins can be identified and their function understood as pertaining to PTSD. However using these techniques one can only study a single protein at a time. Proteomics offers a powerful alternative whereby one can investigate the alterations in several proteins simultaneously. In doing so, one can unravel the contributions of a myriad of proteins in the establishment of complex disease states such as PTSD (Griffin & Aebersold, 2001). In addition proteomics may also provide the platform to identify new biological markers involved in fear extinction, and these may act as novel targets for PTSD therapeutics.

1.3 Research Problem

Current literature shows that the treatment of PTSD remains problematic. One of the reasons is that the pathophysiology of the disorder is still not completely understood. Nevertheless a number of novel drugs are being evaluated for the management of PTSD, DCS being one of them. While DCS shows promising results as a possible remedy for

PTSD, its exact mechanism of action is unclear. Finally few clinical and animal studies have investigated the behavioural and neurobiological effects of PTSD in adolescents, and subsequently there is a huge need for comparative studies between this age group and adults.

1.4 Research Question

The overall research question was to compare some characteristics of PTSD and to see whether infusion of DCS has any effect on fear conditioning and the extinction of fear in adolescent versus adult rats.

1.5 Research Aims

In line with the research problem and question the following aims were examined in our investigations of adolescent and adult rats:

- Establish and optimize a rat model of PTSD based on the methodology of Siegmund and Wotjak (2007),
- Assess the role of certain specific neurochemical parameters in PTSD,
- Determine whether intrahippocampal DCS infusion can reverse the PTSD phenotype as displayed by our adolescent and adult PTSD model,
- Identify other proteins that may be important in establishing PTSD

1.6 Specific Research Questions

The following specific research questions with regards to our PTSD rat model were formulated:

- Does brief, intense exposure to electric footshock produce a PTSD phenotype in rats,
- What are the roles of BDNF, nNOS and NMDARs in the development of the PTSD phenotype,
- Does intrahippocampal infusion of DCS enhance fear extinction, BDNF expression and nNOS levels in the HC,
- Will hippocampal NMDARs be upregulated in response to fearconditioning, and if so, will intrahippocampal infusion of DCS normalize this upregulation,
- Do adolescent and adult rats exhibit similar fear extinction features, and
- What other proteins may be relevant in the development of PTSD and the effects of DCS in adolescent and adult rats.

1.7 Hypothesis

We hypothesized that exposure to brief, intense electric footshocks will lead to the development of PTSD-like symptoms in rats. These symptoms will be associated with a decrease in BDNF expression, and an upregulation of NMDARs and nNOS in the HC. DCS infused into the HC will reverse these effects. It was further hypothesized that the neurobiology of PTSD-like symptoms in adolescent rats was dissimilar to that of adult animals.

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Chapter 2

Review of Posttraumatic Stress Disorder

2.1 Introduction and historical overview of PTSD

PTSD is a relatively new diagnosis, yet people have been experiencing extremely stressful, potentially life-threatening events for centuries. Traumatic events have been shown to result in feelings of emotional tension, cognitive impairment, somatic complaints and conversion phenomena. Throughout the years, various names have been used to describe the traumatic manifestations. Railway spine, stress syndrome, shell shock, battle fatigue (Russel, 1919), traumatic war neurosis, and posttraumatic stress syndrome (DSM III, APA, 1980) were a few of the names used. Descriptions were mostly based on the common stressor at the time namely, war stress.

During the 1800s soldiers were diagnosed with “exhaustion” following their body’s natural shock reaction to the intense and frequently repeated stress of battle (Russel, 1919). This battle fatigue was characterized by mental shutdown in both individual and groups exposed to trauma. Official documents of the Crimean War (1850’s) referred to cases of ‘irritable heart’; a ‘form of cardiac malady common among camp soldiers’. Soldiers reported a range of physical symptoms as well as phobias, such as nightmares and nervousness (Skerritt, 1983). At the time, war stress was recognized as the only psychological trigger for PTSD (Kardiner & Spiegel, 1947; Magruder & Yeager, 2009). However, soldiers were not the only people experiencing these debilitating symptoms in reaction to a traumatic external event.

Stresses other than combat have been recognized in the development of PTSD. Victims of natural disasters and exceptionally threatening events such as tsunamis (Thavichachart et al., 2009; Pyari et al., 2012), terrorist attacks (North et al., 1999; Yehuda, 2002), rape (Foa et al., 1991; Schnurr et al., 2002) and violent abuse (Breslau et al., 1998; Creamer et al., 2001; Norris et al., 2003; Zlotnick et al., 2006) were found to exhibit PTSD symptoms. Even witnessing a traumatic event was enough to elicit a clinically diagnosable stress response in some people. Considering these findings, the interest shifted from war stress to the stress of other catastrophic disasters and human brutality. The trauma experienced by these survivors were found to be immeasurable and long lasting - placing them at high risk of behavioural and emotional readjustment problems.

2.2 Terminology, symptoms and diagnosis of PTSD

In 1978, the term posttraumatic stress disorder was coined. A formal diagnosis of PTSD was introduced during 1980 in the Diagnostic and Statistical Manual (DSM)-III (APA, 1980). In 2000, the APA revised the PTSD diagnostic criteria noted in the fourth edition of the DSM-IV. The revised criteria focused on diagnosis and management, including the detection and treatment of PTSD co-morbidities (DSM-IV-TR). PTSD as defined in the DSM IV-TR (APA, 2000) requires exposure to an extremely traumatic event which threatens the life or physical integrity of oneself or others. The person exhibits intense fear, horror or helplessness (Asmundson & Taylor, 2009). These acute peri-traumatic emotional reactions and accompanying physiological reactions have been shown to be predictive of PTSD (Shalev et al., 2000; Bryant, 2003). In 2013, the DSM-IV-TR was superseded by the DSM-5 (APA, 2013). The DSM-5 re-classified PTSD from an anxiety disorder to a trauma- and stress-related disorder which is characterized by an additional diagnostic symptom cluster i.e. negative cognitions and mood (Friedman et al., 2011; APA,

2013). Furthermore, Criterion A2 (APA, 2000) which required specific subjective emotional reactions to trauma, was eliminated in the DSM-5 (APA, 2013). Removal of Criterion A2 was in lieu of PTSD being more frequently diagnosed among military and emergency personnel who were trained not to express emotional reactions while on scene. Thus empirical support for the usefulness and prognostic validity of this Criterion was found to be lacking (Friedman et al., 2011). The DSM-5 therefore focuses on the psychological reactions as opposed to the emotional reactions exhibited in response to PTSD. Two subtypes, namely PTSD preschool subtype and PTSD dissociative subtype, were also recognized and included in the DSM-5 diagnosis of PTSD (APA, 2013).

Table 2.1: Diagnostic criteria of PTSD

APA Manual	Criteria
DSM-5 (APA, 2013)	<ul style="list-style-type: none"> A. Exposure to a traumatic event (e.g. sexual assault) B. Persistent re-experience (e.g. flashbacks, nightmares) C. Persistent avoidance of stimuli associated with the trauma (e.g. avoidance of experiences that they fear will trigger flashbacks and re-experiencing of symptoms, fear of losing control) D. Negative alterations in mood and cognition (e.g. feeling detached or disconnected, memory problems) E. Persistent symptoms of heightened arousal (e.g. difficulty falling or staying asleep, anger and hypervigilance) F. Duration of symptoms for more than 1 month G. Significant impairment in social, occupational, or other important areas of functioning (e.g. problems with work and relationships) H. Attribution (e.g. medication, substance use or other illness is not the cause of disturbance)

2.3 Clinical symptoms of PTSD

Clinicians look for four main clusters of symptoms when diagnosing PTSD. The symptoms include re-experiencing, avoidance, hyper-arousal and negative mood and cognition (APA, 2013; Asmundson & Taylor, 2009; Khozhenko, 2009).

2.3.1 Re-experiencing symptoms

People suffering from PTSD tend to experience highly emotional, intrusive memories and recurrent, distressing nightmares. These re-experiencing symptoms create the feeling of reliving the traumatic event and may persist indefinitely (Kenny et al., 2009). PTSD patients report mental and physical distress which manifests as disturbed sleep, daytime hyper-arousability, various infections due to immune system dysfunction and other bodily complaints such as headaches (Hakamata et al., 2007).

2.3.2 Avoidance symptoms

People with PTSD unconsciously avoid associations with the traumatic event. They tend to distance themselves from people, thoughts, feelings or situations connected with the traumatic event which ultimately manifests in social withdrawal.

2.3.3 Hyper-arousal symptoms

PTSD patients report difficulty in concentrating, being easily startled and highly irritable, experiencing outbursts of anger and exaggerated wariness (Orth & Maercker, 2009).

2.3.4 Negative thoughts and feelings or mood

The decline in thought patterns or mood of PTSD patients, comprise of a misguided sense of blaming oneself or others, an inability to remember key features of the event and intense emotions related to the trauma such as horror or sadness. These negative thoughts and feelings manifest in limited emotions, feeling disconnected from others and a reduced interest and participation in important activities (Lanius et al., 2003; Milad et al., 2009).

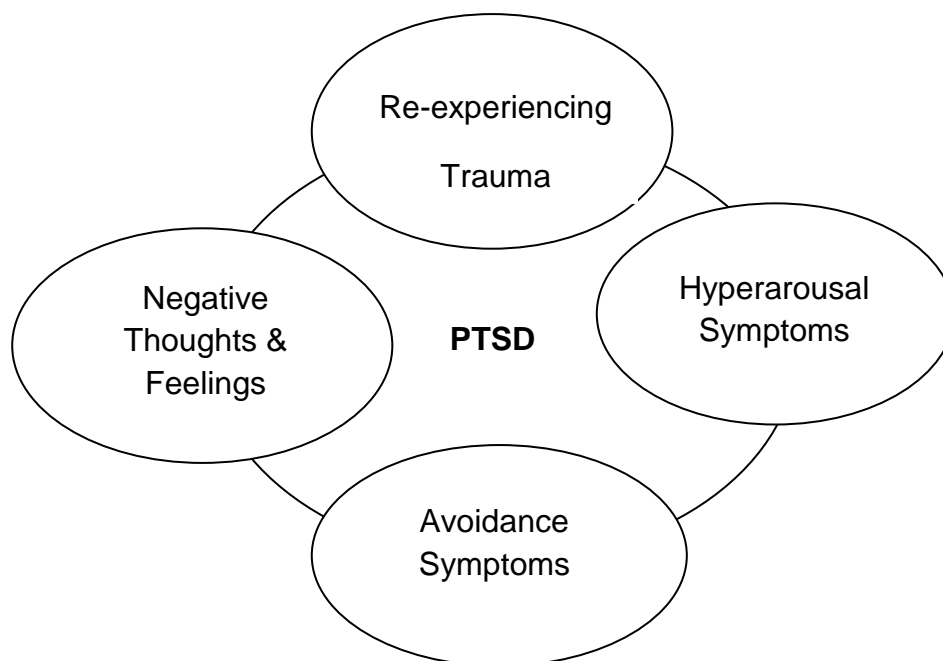


Figure 2.1 Characteristics of PTSD

2.4 PTSD diagnosis

Three broad risk factor categories were identified namely, pre-trauma, peri-trauma and post-trauma. These risk factors, discussed below, are applicable to anyone at any stage of their lives, making PTSD an unpredictable yet common source of distress (Schiraldi, 2000).

2.4.1 Pre-trauma factors

Pre-trauma factors can be divided into two main categories consisting of individual-related and family-related risk factors.

2.4.1.1 Individual-related pre-trauma factors

Individuals are particularly susceptible to recurrent bouts of PTSD if they were previously exposed to trauma, especially if PTSD developed. This could be due to the recall and re-experiencing of the prior trauma (Matsakis, 1992; Karunakara et al., 2004; Schiraldi, 2000). Experiencing life stressors that are not deemed traumatic (eg. divorce, job loss) may still weaken a person's defence against trauma (Schiraldi, 2000). In addition, poor coping skills and certain personality traits such as pessimism have been shown to increase a person's chance to develop PTSD. However, it should be noted that both coping skills and personality are modifiable (Schiraldi, 2000). Twin studies have also demonstrated a role for genetic factors in the aetiology of PTSD (Sullivan et al., 2000; Rhee & Waldman, 2002; Koenen et al., 2008). These studies implicated that PTSD is partly heritable.

Moreover, adverse stimuli have been shown to damage brain structures and dysregulate neurochemical pathways. Specifically, functional and structural alterations in the HC were observed which impacts on learning and memory systems (Nutt, 2000). Dysregulation of neurotransmitter pathways cause long-term changes to synaptic memory. Consequently, the encoding of memories become excessive which may explain the re-experiencing (eg. flashbacks) symptoms of PTSD (Nutt, 2000). The victim relives the initial trauma through repeated experiences that can be just as distressing as the original (McFarlane, 2000).

Another well researched PTSD risk factor is gender. Studies report men to have experienced more traumatic events throughout their life, with the exception of sexual violence. However, women are twice as more likely to develop PTSD (Breslau et al., 1999; Perkonig et al., 2000).

2.4.1.2 Family-related pre-trauma factors

A disturbed childhood environment, where the child was neglected or mistreated, is associated with PTSD (Streimer et al., 1985). Other examples include poor parent-child relationships, absence of the parent and even a history of parental substance abuse (Karunakara et al., 2004). Another risk factor implicated in PTSD development, is the presence of pre-existing psychiatric conditions (Kessler et al., 1995). In particular, comorbidity with other psychiatric disorders, usually depression, has been reported in 80% of adolescents presenting with PTSD (McFarlane, 1989). Similarly, parents with a history of PTSD predispose other family members to develop this disorder (Durand, 2006).

2.4.2 Peri-trauma factors

Victims who, at the outset of a traumatic event feel responsible for the trauma or believe they could have done more to prohibit it from occurring, dissociate themselves and feel isolated; tend to experience more adverse symptoms. Schiraldi (2000) states that these negative thought patterns disconnect the victim from the event and may cause undue feelings of shame. Other peri-trauma factors include the proximity to and severity of the trauma. The proximity to trauma is directly proportional to PTSD development (Pynoos et al., 1987). Survivors from an elementary school sniper shooting, who were closest to the playground where many were injured and killed, reported greater PTSD prevalence and

symptoms (Pynoos et al., 1987). It has also been demonstrated that brutal trauma more frequently results in PTSD, specifically chronic cases (Durand, 2006). On the other hand for low-level trauma exposure, an individual's vulnerabilities are more likely to determine PTSD development (Durand, 2006). Furthermore, the type (eg. rape, earthquake) and nature (eg. sudden, recurring) of the trauma may interact with the trauma severity to increase the victim's vulnerability to PTSD development.

2.4.3 Post-trauma factors

The level of social and family support is crucial to PTSD development. Ignorance of the psychiatric disorder and lack of support for PTSD sufferers result in social integration problems, low self-esteem and feelings of anger and alienation (Frye & Stockton, 1982). Some people who previously experienced trauma may be emotionally unavailable (Briere, 2004) and unable to provide support for the victim during distressing times. In addition, they may teach maladaptive coping methods to the victim due to their own debilitating experience. Disbelieving and rejecting the victim's story (particularly rape victims), secondary victimization (subtly blaming victim) and lack of treatment (intentional or ignorant) only serves to intensify the victim's distress (Schiraldi, 2000).

2.5 Epidemiology and neurobiology of PTSD

2.5.1 Aetiology, prevalence and demographics of PTSD

PTSD is the fourth most common psychiatric disorder (Breslau et al., 1991; Resnick et al., 1993; Kessler et al., 1995). Often exposure to a combination of both physical and psychological trauma is believed to cause PTSD (Yehuda, 1997; Ehler et al., 2001; Yehuda, 2002; Yehuda et al., 2004; Shea et al., 2005). The intensity and length of the

trauma are generally seen as essential aetiological factors in the individual's response to the stress. Globally, 50-90% of people encounter trauma over a lifetime, yet only a small fraction, about 7%-10% of the population develops full PTSD (Breslau et al., 1998; Kessler et al., 2005; Breslau, 2009). Various epidemiological studies have found that traumatising events (e.g. domestic violence, rape, car jacking) known to precipitate PTSD (Foa et al., 1991; Breslau et al., 1998; Creamer et al., 2001; Schnurr et al., 2002; Norris et al., 2003; Zlotnick et al., 2006) are a common occurrence in South Africa (Peltzer, 1999; Seedat et al., 2000; McGregor et al., 2002; Carey et al., 2003; Malose, 2004), with about 75% of South Africans reported to have experienced at least one traumatic event in their lifetime (Kaminer et al., 2008). Given South Africa's exceptionally high rates of domestic and criminal violence (South African Police Service, 2014) in addition to the past violent human rights abuses (Dowdall, 1992; Friedman, 2000, Gobodo-Madikizela 2004), South Africans are considered to be at high risk of developing PTSD. Women demonstrated a twice as higher incidence rate for PTSD (10.4%) when compared to men (5%) (Kessler et al., 1995). Recent research also found that adolescents were more susceptible than adults in developing PTSD, with a 22% PTSD prevalence rate noted amongst the South African youth (Seedat et al., 2004).

As previously mentioned, witnessing or experiencing traumatic natural (eg. fires, floods) or human-made (eg. kidnapping, terrorist attacks) events can result in the development of PTSD symptomatology (APA, 2013). Moreover, dysfunctional extinction of fear memories increased the severity and persistence of PTSD symptoms (Bremner et al., 1996). These symptoms become so debilitating that PTSD sufferers become a health and economic burden to society (Zatzick et al., 1997; Mendlowicz & Stein 2000). The high morbidity of PTSD prompted an increased awareness to recognize, understand and treat this psychiatric disorder. During the 1990s tremendous advances were made in molecular

biology, neuropharmacology and clinical neuroimaging technology. This progress enabled the identification of neurocircuits and numerous neurotransmitter systems underlying fear and anxiety behaviour.

2.5.2 Neurocircuitry and neurochemistry of PTSD

The development of mood and anxiety disorders is highly correlated with stressful life events. Research has suggested that the stress response is crucial to survival and maintenance of homeostasis. However, a dysregulated stress response may be a predisposing factor for these affective disorders (McEwen et al., 2002). In fact, a primary feature of PTSD is the failure to initiate a normal stress response which partly results from hypothalamic–pituitary–adrenal-axis (HPA) dysregulation. The HPA axis maintains homeostasis by coordinating the hormonal response to stress and activating the locus coeruleus-noradrenergic (LC-NA) system (Claes, 2004). Several other neurobiological systems have also been implicated in the aetiology of PTSD.

2.5.2.1 Neurocircuitry of PTSD

Rauch et al. (1998) outlined a neurocircuitry model for PTSD. This study highlighted the interactions of the amygdala with the ventral/medial prefrontal cortex and HC in the processing of emotions (Rauch et al., 1998). Numerous human brain imaging studies in PTSD patients (Sehlmeyer et al, 2009; Rauch et al, 2006) also confirmed the role of these limbic structures in the mediation of fear conditioning and extinction (Fredrikson et al., 1976; LaBar et al., 1998; Büchel et al., 1999; Milad & Quirk, 2002; Morris & Dolan, 2004; Phelps et al., 2004; Cheng et al., 2003; Myers & Davis, 2002; Knight et al., 2004; Quirk & Mueller, 2008). Poor amygdaloid-prefrontal cortex and hippocampal-prefrontal cortex signalling was postulated to underlie contextual memory deficits in PTSD that result in poor

contextual control of conditioned responses (Acheson et al, 2011). Complementary evidence from animal studies has confirmed these limbic structures in fear conditioning, associative learning and extinction (Morgan et al., 1993; Sotres-Bayon et al., 2004; Corcoran & Maren, 2004; Bouton, 2004; Bouton et al., 2006). Specifically, rodent studies have successfully delineated the neurocircuitry underlying fear conditioning and extinction (Lu et al, 2001; Milad & Quirk, 2002; Morgan & LeDoux, 1995; Morgan et al, 1993; Walker et al, 2002).

The majority of research on mood and anxiety disorders has focused on the amygdala and its connections to the prefrontal cortex. PTSD drug treatment assessment has mainly investigated the effects of drug by systemic or intra-amygdalar administration. Recently, the HC emerged as a region of interest given its significant role in learning, memory and the processing of emotional behaviour (Kjelstrup et al., 2002; Bannerman et al., 2004; McHugh et al., 2004).

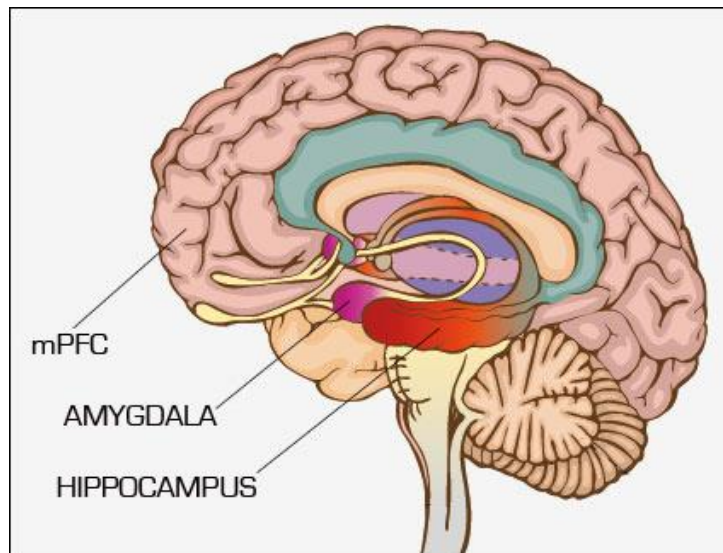


Figure 2.2: The neurocircuitry involved in PTSD

2.5.2.2 The role of the hippocampus in PTSD

The HC has been extensively studied (Cajal 1911; Raisman et al., 1965, 1966; Meibach & Siegal 1975, 1977a,b; Swanson & Cowan 1975, 1976; Steward 1976; Swanson et al., 1981). Studies proposed a region-specific function for the HC (Moser et al., 1993; Richmond et al., 1999; Bannerman et al., 2004; McHugh et al., 2004; Zhu et al., 2006; Oomen et al., 2010; Korosi et al., 2012), with the dorsal hippocampus (DHC) being preferentially involved in spatial learning (Moser et al., 1993), while the ventral hippocampus (VHC) is crucial for anxiety-like behaviour (Richmond et al., 1999; Bannerman et al., 2004).

Considered as one of the few areas able to produce neurons throughout the life of humans and animals (Eriksson et al., 1998), the HC is prone to be influenced by different environmental stressors (Gould et al., 1998; Lemaire et al., 2000). Psychological and physical stressors (Lemaire et al., 2000; Czeh et al., 2002) may influence hippocampal neurogenesis and this may impact on the structure of the HC. For instance early life trauma has been suggested to manifest as psychopathologies, such as anxiety disorders, later in life (Kendler et al., 1992; Heim & Nemeroff, 2001) and adverse events such as these have been demonstrated to result in hippocampal volume loss (Kaufman et al., 2000; Neumeister et al., 2005; Kitayama et al., 2005) as well as hippocampal neuron loss (Duman et al., 1997; Duman et al., 1999; McEwen, 1999). A number of molecular underpinnings have been posed for the reduced hippocampal volumes including decreased hippocampal neurogenesis (DeCarolis & Eisch 2010; McEwen, 1999), gliogenesis, enhanced apoptosis (Czeh & Lucassen, 2007) and oxidative stress (Diehl et al., 2012).

Extensive anatomical and neuroimaging studies of the HC in PTSD patients have reported abnormalities in this limbic region (Elzinga & Bremner, 2002; Quirk & Mueller, 2008). For example Bonne et al. (2008) indicated a significant reduction in posterior hippocampal volume in PTSD patients compared to controls, with no significant differences in the size of the anterior HC (Bonne et al. 2008). Furthermore, human twin studies reporting on stress-associated hippocampal loss suggested that hippocampal volume loss is an indicator of vulnerability for PTSD development as opposed to being a consequence of the traumatic stress (Gilbertson et al., 2002; Pitman et al., 2006). Animal studies have complemented the clinical neuroimaging studies, indicating a role for the HC in fear conditioning and extinction (Anagostaras et al. 2001; Phillips & LeDoux, 1992). A study by Yang et al. (2011) suggested a distinct role for the DHC in the reconsolidation process of fear memories. Thus hippocampal volume loss may be caused by the trauma itself (Matrisciano et al., 2011; Liu et al., 2011), leading to learning and memory abnormalities as that underpinning PTSD. A clinical study by Felmingham and colleagues (2009) supported the negative correlation between hippocampal volume and PTSD duration.

Interestingly findings between adults and youth with PTSD appear contrasting. Studies in adults demonstrated smaller hippocampal volumes compared to healthy control subjects (Bremner et al., 1995, 1997; Gurvits et al., 1996; Stein et al., 1997) or trauma-exposed subjects without PTSD (Gurvits et al., 1996). Conversely, studies in children and adolescents with PTSD fail to show hippocampal volume reduction (DeBellis et al., 1999; Carrion et al., 2001). It is therefore evident that further research is needed to fully understand the neurobiology of PTSD in the young and the adult.

2.5.2.3 The glutamatergic system and PTSD

Multitudes of neurotransmitter systems have been implicated in PTSD. Dopamine, epinephrine, acetylcholine, opioid peptides, serotonin and glutamate are a few of the neurotransmitters demonstrated to play a role in PTSD. However in recent years much focus has been on the role of the glutamatergic system.

Glutamate-mediated synaptic transmission was found to be critical for the normal functioning of the nervous system. Glutamate can bind to one of two types of receptors, namely ionotropic or metabotropic receptors. Since ionotropic receptors are mainly involved in rapid transmissions as is required for processes such as learning and memory, the present overview will only concentrate on the role of these receptors in PTSD.

Three types of glutamate ionotropic channels have been identified, namely kainate, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors. In particular, the glutamatergic amygdala and hippocampal NMDARs were suggested to mediate the associative learning and behavioural processes underlying PTSD (Heresco-Levy & Javitt, 1998). Conversely, dysfunctional NMDARs have been implicated as a crucial neurobiological component of PTSD. Studies have demonstrated an increased release of glutamate in response to stressful situations (McEwen 1999; Sapolsky 2000). This elevation in synaptic glutamatergic levels leads to overactivation of NMDARs, resulting in neurotoxicity (Chambers et al., 1999). This neurotoxicity has been forwarded as a possible explanation for the degree of hippocampal atrophy (Elzinga & Bremner, 2002) and concomitant cognitive deficits (Bremner, 1999; Vermetten et al., 2003).

2.5.2.3.1 *N*-methyl-*D*-aspartate system

Recent studies have implicated the glutamatergic NMDA system, specifically the amygdala and HC, in fear extinction (Walker et al., 2002; Ledgerwood et al., 2003, 2004; 2005; Yang & Lu, 2005; Bevilaqua et al., 2005; Szapiro et al., 2003; Yamamoto et al., 2001). Long-lasting behavioural effects of traumatic events were shown to be mediated by NMDAR activation (Sziray et al., 2006), with increasing evidence supporting the critical role of hippocampal NMDARs in these fear-related processes (Falls et al., 1992; Baker & Azarloza, 1996; Sotres-Bayon et al., 2007). These receptors have been implicated in neural changes supporting fear conditioning and loss of fear through fear extinction. For example, NMDAR-deficient mice were found to be less sensitive to stress as indicated by data generated by tools such as the light/dark box, the forced swim test and the elevated plus-maze, assessing the emotionality of animals (Miyamoto et al., 2002).

Under conditions of normal activation, NMDARs have been shown to be critical for neural plasticity underlying learning (Bear & Malenka, 1994). Thus, experience-dependent forms of neuronal plasticity for example, learning and memory, are thought to be NMDAR-dependent (Davis et al., 2006). Interestingly, the implication of the glutamatergic system in fear extinction originated from studies of NMDAR antagonists, which were found to block fear extinction (Falls et al., 1992; Myers & Davis, 2002). Given this finding, the question arose as to whether pharmacological enhancement of the NMDAR might facilitate extinction. Indeed, one such NMDAR partial agonist, DCS, was found to facilitate fear extinction (Walker et al., 2002) and enhance receptor efficacy (Norberg et al., 2008).

A significant difference exists between the immature and mature rat brain regarding the composition of NMDAR complexes. The developing HC expresses NMDARs as diheteromers (NR1/NR2B) and triheteromers (NR1/NR2B/D) respectively (Pina-Crespo &

Gibb, 2002), while in the adult HC NR2A and NR2B (in pyramidal cells) and NR2C subunits (in different interneurons) are mostly found (Monyer et al., 1994). The various combinations of NMDAR subunits determine the composition of NMDARs and this impacts on the overall functionality of the receptor. For instance elegant studies by Cavara and Hollman (2008) showed how NMDAR subunit NR3 altered the sensitivity of the NMDAR to magnesium (Mg^{2+}) as well as changed the receptor's calcium (Ca^{2+}) permeability when compared to NMDAR that do not have the NR3 subunit in their structure. Moreover this composition of NMDARs has been shown to change with time as the poor performance of aged animals in tasks requiring cognitive skills, when compared to young animals, was attributed to changes in NMDAR subunit structure (Magnusson et al., 2010). In addition to these findings depicting the importance of NMDAR subunit composition in the determination of brain function, are other investigations reporting the relevance of NMDAR number expressed on the neuron membrane. For example reviews by Stephenson et al. (2008) and Groc et al. (2009) highlight the essence of NMDAR trafficking and surface expression in normal brain function and elaborate on their role in the development of psychiatric disorders, especially those where learning and memory deficits are prominent features.

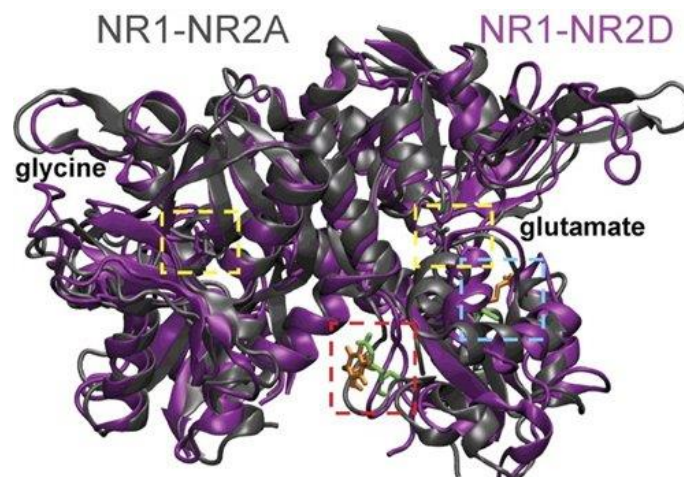


Figure: 2.3: A NMDA receptor with subunits 1 and 2

2.5.2.3.2 The role of NMDA receptors in Long Term Potentiation: relevance to Learning and Memory

Learning and memory are two cognitive processes that are impaired in PTSD (Friedman 1997; Vermetten & Bremner, 2002). Long term potentiation (LTP), a form of neural plasticity, is widely believed to be one of the main neural mechanisms by which memory is stored in the brain (Hölscher 1999; Bliss & Collingridge 1993). As such a number of studies have examined the involvement of the glutamatergic system in LTP.

Some forms of LTP have been demonstrated to be dependent on NMDAR activity emphasizing the integral participation of this receptor system in memory consolidation (Quartermain et al., 1994; Lanthorn 1993). Interestingly, acquisition of fear has not been shown to be affected by NMDA agonism so it may be that only extinction, or learning to inhibit a response that is no longer adaptive, is affected by the NMDA system (Davis, 2005).

Hippocampal synaptic plasticity, involving repeated activation of NMDARs and L-glutamate release (Kato et al., 1998), is regarded as a cellular model of learning and memory (Bliss & Collingridge, 1993). LTP induction in the CA1 but not other hippocampal areas (Diamond et al., 2007; Joëls, 2008; Kim & Diamond, 2002) was shown to require the simultaneous activation and postsynaptic depolarization of NMDARs in hippocampal pyramidal cells. NMDAR activation results in an excitatory postsynaptic potential which opens the ion channel allowing an influx of Ca^{2+} into the cell. This increased Ca^{2+} acts as a second messenger in numerous signalling pathways. However the ion channel only opens when glutamate binds the receptor and Mg^{2+} blocking the channel is removed in order to depolarize the cell. In addition to glutamate, either glycine or the co-agonist D-serine is

required for NMDAR co-activation and channel opening (Figure 2.4). Because of this requirement of co-activation, some recent rodent studies investigated the potential of co-stimulators to facilitate LTP and fear extinction (Myers et al., 2011). As such a few laboratories have studied the partial agonist DCS, which binds to the glycine modulatory site on the NMDAR complex (Carter, 1992; Kemp & Leeson, 1993; Sheinin et al., 2001), in neuroplasticity and fear memory consolidation.

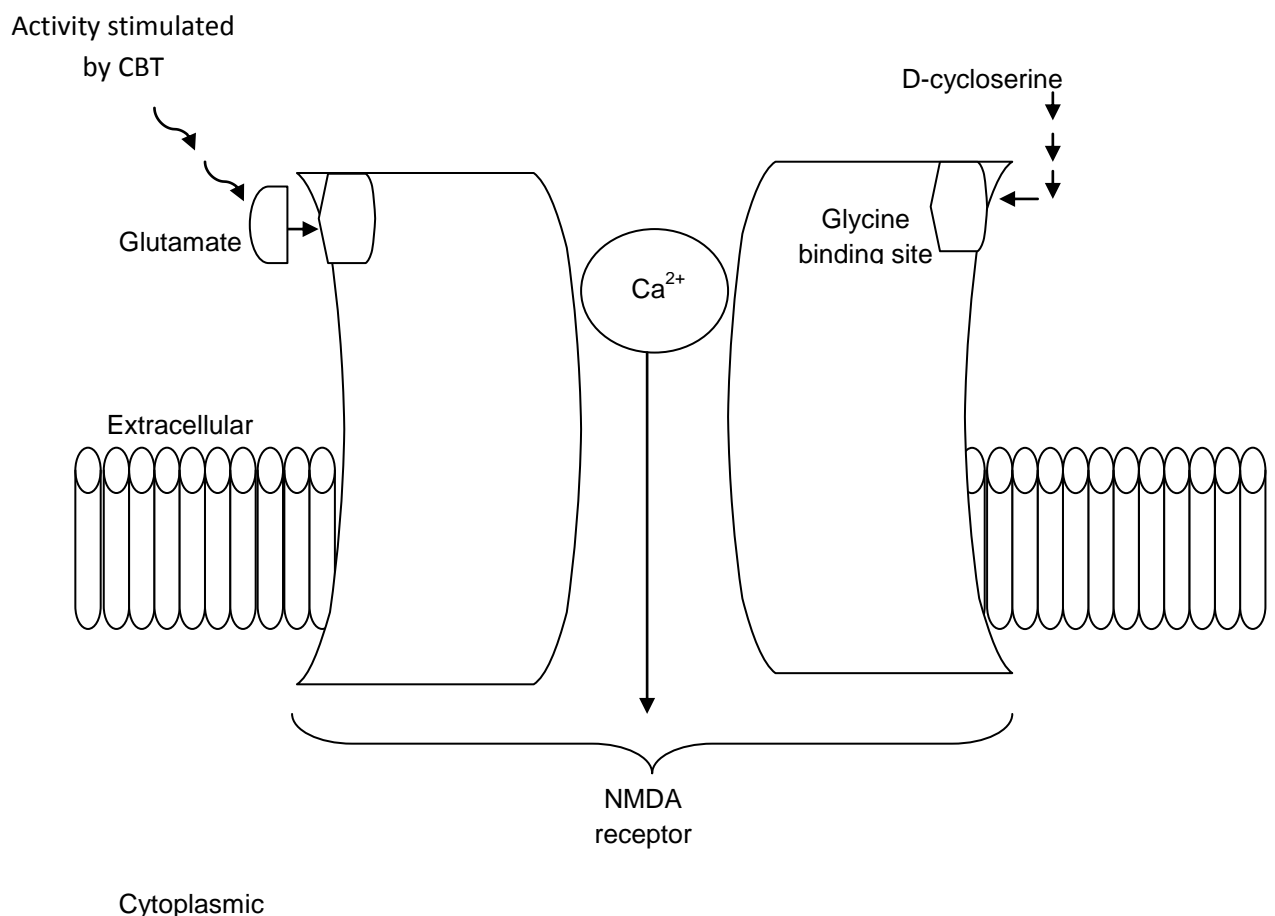


Figure 2.4: Important components in a glutamatergic synapse (adapted from American Journal of Psychiatry; 165:293-296, 2008)

A number of investigations have confirmed hippocampal aberrations in PTSD patients (Kitayama et al., 2005; Bremner et al., 1995; 2003; Gurvits et al., 1996; Vythilingam et al., 2005) compared to controls. Of note is the significant reduction in the dorsal/posterior

hippocampal volume with no differences recorded in the ventral/anterior hippocampus (Bonne et al., 2008). This region-specific feature of the HC has a strong influence on how the animal relates to processing of emotional behaviour (Kjelstrup et al., 2002; Bannerman et al., 2004; McHugh et al., 2004). It is now accepted that the DHC mainly has a role in memory and a preference for spatial learning while the VHC is crucial for anxiety-like behaviour (Brown et al. 1999). Since the HC is rich in NMDARs, structural changes to this brain area may precipitate alterations in NMDAR number. As mentioned earlier NMDARs function as ion channels that allows the influx of Ca^{2+} into the neuron. Therefore aberrations in NMDAR will necessarily lead to dysregulation in intracellular Ca^{2+} concentrations and eventually affect the functioning of intracellular Ca^{2+} dependent enzymes. One example of such an enzyme is nNOS. Some lines of evidence have indicated that the NMDA–nNOS pathway may play an important role in anxiety-related behaviours (Podhorna & Brown 1999; Masood et al. 2003).

2.5.2.3.3 Neuronal Nitric Oxide Synthase (nNOS)

Good evidence exists for an involvement of the NMDA system in the pathology and treatment of stress and anxiety (Krystal et al., 2002; Stewart & Reid, 2002). For example using an animal model of PTSD, Oosthuizen et al. (2003) could show marked changes in both hippocampal NMDARs and HC nitric oxide synthase (NOS) activity. More specifically, activation of the NMDAR results in a Ca^{2+} influx which leads to nNOS activation to produce the retrograde messenger, NO (Snyder, 1992). Behavioural evidence suggests a role for NO in synaptic plasticity and fear memory formation processes particularly during late phase LTP (Lu et al., 1999; Arancio et al., 2001; Puzzo et al., 2006) and long-term memory (LTM) consolidation (Medina & Izquierdo 1995; Rickard et al., 1998; Ota et al., 2008). Studies of the site-specific effects of NO signalling involving pharmacological blockade of

this enzyme demonstrated that NO impairs cued (Schafe et al., 2005) and contextual (Resstel et al., 2008) fear conditioned learning.

2.5.2.3.4 Brain-derived neurotrophic factor (BDNF)

Neurotrophins are essential for the differentiation and survival of neuronal cells. Not surprisingly clinical and preclinical research have implicated nerve growth factor (NGF) (Levi-Montalcini, 1996), neurotrophin-3 (NT3) (Hohn et al., 1990) and BDNF (Barde et al., 1982) in various mental disorders (Calabrese et al., 2009; Casey et al., 2009; McEwen, 2008; Kauer-Sant'Anna et al., 2007; Duman & Monteggia, 2006; Roceri et al., 2002). Of the neurotrophins BDNF is the most prevalent and best characterized in terms of its role in normal and abnormal brain function (Lohof et al., 1993; Levine et al., 1995, 1998; Kossel et al., 2001).

BDNF has been implicated in PTSD pathophysiology with clinical and basic studies supporting the idea that decreased hippocampal BDNF levels mediate trauma behavioural arousal (Shirayama et al., 2002; Dell'Osso et al., 2009; Hauck et al., 2009, 2010). A rodent study by Uys (2006) observed decreased BDNF levels in the dorsal, but not ventral hippocampus following adolescent trauma exposure followed by re-stressing in adulthood. This observation was supported by another rodent study where rats displaying PTSD-like symptoms in response to trauma, also exhibited a down regulation in hippocampal BDNF messenger ribonucleic acid (mRNA) (Kozlovsky et al, 2007). The involvement of BDNF in PTSD was further confirmed by a genetic study by Heldt et al. (2007). These authors demonstrated how a specific deletion of the hippocampal *BDNF* gene led to a decrease in fear extinction as well as impaired learning. These findings and others emphasize the role of the hippocampal BDNF in the extinction of contextual fear conditioning (Kalisch et al., 2006; Ji & Maren, 2007; Milad et al., 2007; Lang et al., 2009).

Lessman and colleagues (2003) have shown that BDNF is synthesized as a precursor protein which is cleaved into pro-BDNF and then eventually cleaved into mature BDNF. It has been suggested that different intracellular signalling pathways are activated by pro-BDNF and mature BDNF (Woo et al., 2005; Matsumoto et al., 2008; Yang et al., 2009). Studies of the low-affinity neurotrophin receptor p75^{NTR} is activated by pro-BDNF (Roux & Barker, 2002; Lessmann et al., 2003) whereas mature BDNF binds to and signals through the tropomyosin related kinase (Trk) receptors (Lessman et al., 2003; Segal, 2003), specifically TrkB. Upon binding, TrkB receptors are phosphorylated at tyrosine residues which lead to activation and regulation of three major downstream pathways. The three signalling transduction pathways are inositol triphosphate (IP3) dependent Ca²⁺ release/phospholipase C γ pathway, which activates protein kinase C; the phosphatidylinositol-3 kinase (PI-3K) pathway, which leads to the activation of serine/threonine protein kinase B; and the Ras/mitogen-activated protein kinase (MAPK) cascade or extracellular signal related kinase (ERK) pathway, which stimulates several downstream effectors (Segal, 2003; Huang & Reichardt, 2003). The ERK/MAP kinase pathway has been implicated in associative learning and contextual fear memory formation (Atkins et al., 1998; English & Sweatt, 1996). The PI-3K pathway has been associated with the forkhead and poly adenosine diphosphate ribose polymerase (PARP) proteins (Zheng et al., 2002; de la Monte et al., 2000) and recognized as being crucial for cell survival (Huang & Reichardt, 2003; Brunet et al., 2001). This pathway has also recently been implicated in the activation of hippocampal p42 MAPK (ERK2) during the retrieval of contextual fear memories (Chen et al., 2005).

In addition to activating intracellular signalling cascades, evidence also suggests that BDNF directly activates voltage-gated sodium channels in order to mediate rapid depolarization of target neurons (Blum et al., 2002) e.g. increasing NMDAR currents

(Levine et al., 1998). Furthermore, an interaction between BDNF and glutamatergic receptors on synaptic plasticity has been demonstrated, whereby BDNF has been shown to heighten glutamate release, enhance ionotropic glutamate NMDAR activity and increase phosphorylation of NMDAR subunits (Minichiello, 2009). In a study employing a PTSD model to examine the impact of prior stress experience on subsequent contextual fear memory formation, it was shown that the earlier trauma resulted in enhanced freezing levels that was associated with epigenetic changes to the BDNF gene in the HC (Takei et al., 2011). Collectively these observations point to a notable involvement of BDNF in PTSD, thereby justifying further investigation into the role of this neurotrophic factor in fear extinction.

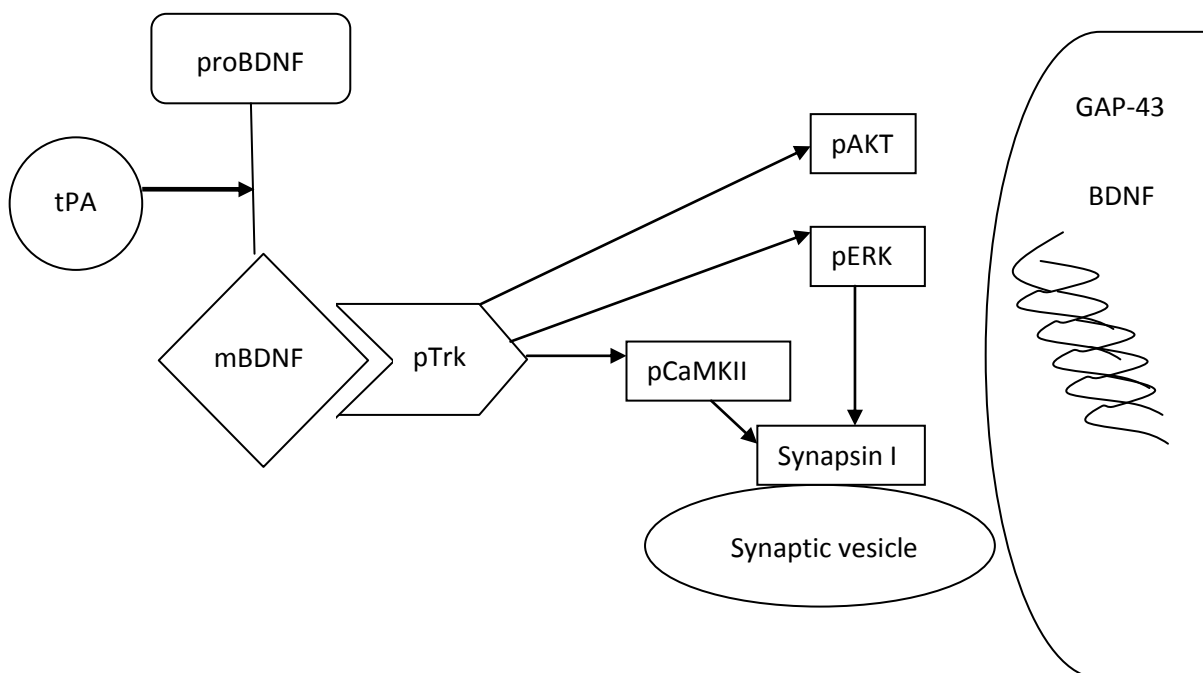


Figure 2.5: BDNF pathway and some of its downstream effectors.

Depiction of the BDNF pathway in the HC. Maturation of pro-BDNF into mature BDNF via cleavage by plasminogen activator (PA). Binding of BDNF to its receptor TrkB and subsequent effects of downstream signalling associated with synaptic growth and cell survival (Adapted from Ding et al., 2011).

2.5.3 Molecular mechanisms of PTSD

Notably, of those exposed to trauma only a subset of individuals present with PTSD (Bresslau et al., 1998). This observation poses an interesting question, namely what protects the majority of people when they are exposed to trauma? This phenomenon was defined by Bonanno in 2004 as resilience i.e. “the ability to maintain a state of normal equilibrium in the face of extremely unfavourable circumstances”. On the contrary, vulnerability can be seen as a predisposition of an individual to be affected by environmental stresses as well as the inability to cope with disasters. Multiple studies have highlighted that genetic factors underlie resilience and vulnerability to PTSD.

Gene-environment (GxE) interactions have been proposed as a crucial determinant in PTSD development (Caspi et al., 2002; Moffitt et al., 2005). This hypothesis has been supported by a number of twin and adoption studies that demonstrated a relationship between a traumatic experience and the risk for developing PTSD as a function of genetic factors (Sullivan et al., 2000; Rhee & Waldman, 2002; Koenen et al., 2008). Furthermore, a mutation study reported on the interaction between a stress-related gene and a risk factor for the development of adulthood PTSD (Nugent et al., 2011). As such the interplay between environment and gene variation of individuals, has been receiving much attention.

It is now accepted that genetic influences, to a certain extent, drive individual differences in PTSD susceptibility. Rather than specific genes, gene variants of BDNF and the serotonin transporter (5-HTT) have been found to account for some of the individual differences in PTSD vulnerability (Plomin et al., 1994; Cicchetti et al., 2007; Craig, 2007; Feder et al., 2009). Some clinical studies have also implicated other molecules like cyclin-dependent

kinase 5 (CDK5) and FK506 binding protein 5 (FKBP5) in susceptibility to develop PTSD (Binder, 2009; Touma et al., 2011). GxE interaction studies further suggest that the genotype of an individual not only imparts risk to environmental trauma, but can also signify resilience. In addition, genetic variants have also been found to interact with several brain and hormonal circuits which mediate the stress response. Interactions such as these are suggested to increase the likelihood of vulnerability or resilience to trauma (Viding et al., 2006). Despite these extensive studies, the molecular pathogenesis underlying PTSD remains unresolved, requiring more investigations into identifying other risk factors that may make a person susceptible to developing PTSD.

Nevertheless a few genes have been linked to brain functions such as fear extinction and learning and memory, rendering them suitable to study in PTSD. A summary of genes that have been implicated to have some role in PTSD is shown in Table 2.2. These genes cover a range of functions that speaks to the pathophysiology of PTSD. These findings therefore support the hypothesis of a genetic component in the development of PTSD.

Table 2.2: A list of genes associated with posttraumatic stress disorder and their related functions

Gene (s)	Function	References
<i>Il1rn, Tacr3</i>	Learning and memory	Oprica et al., 2005; Spulber et al. 2009
<i>Cybb</i>	Increased anxiety-related responses	Nair et al., 2011; Rammal et al., 2008; Hovatta et al., 2005; Masood et al., 2008; Schiavone et al., 2009; Liu et al., 2012
<i>Cyp7b1, Trh, Vim</i>	Impaired contextual fear conditioning	Aguilar-Valles et al., 2007; Wittmann et al., 2009; Lisowski et al., 2013; Thompson and Rosen, 2000
<i>Mmp9</i>	Abnormal cued conditioning behaviour	Nagy et al., 2006
<i>Ncf1</i>	Abnormal central nervous system synaptic transmission	Glotzbach-Schoon et al., 2013
<i>Ctsc</i>	Enhanced contextual conditioning behaviour	Harvey and Shahid, 2011.
<i>Fabp7, Npy, Htr2c, Hspb1</i>	PTSD regulation of interleukin-1 production	De la Fuente et al., 2001; Levite et al., 1998
<i>Spp1, Gpnmb</i>	Nervous system morphology	Selvaraju et al., 2004

2.6 Treatment of PTSD

The abovementioned neurocircuitry and their underlying neural systems led to the development of several treatments for PTSD; ranging from psychotherapies to a limited range of pharmacotherapies. Two main types of psychotherapy have been established, namely eye movement desensitization and reprocessing (EMDR), and cognitive behavioural therapy (CBT) (Bradley et al., 2005; NICE Guidelines, 2005; Bisson et al., 2007; Foa et al., 2009). EMDR is a structured eight-step approach entailing the recall of a traumatic memory while receiving bilateral inputs e.g. lateral eye movement. EMDR is suggested to work on the HC; processing distressing memories and flashbacks. Despite EMDR being a relatively new treatment, its efficacy in reducing PTSD symptoms has been encouraging (Van Etten & Taylor, 1998; Bradley et al., 2005; Seidler & Wagner, 2006). On the other hand CBT is based on regulating the process of fear extinction (McNally, 2007; Otto et al., 2007) by directly exploring the trauma and teaching adaptive cognitive and behavioural management techniques to PTSD patients. CBT is currently the more effective of the two psychotherapies in treating PTSD (Foa et al., 2000). Specifically exposure-based therapy (EBT) is empirically supported and currently the most common PTSD treatment. EBT utilizes extinction-based methods such as safe re-exposure to traumatic aspects in order to create positive associations with the traumatic event (Foa & Kozak, 1986; Schiraldi, 2000; Norton and Price, 2007). EBT is thus procedurally analogous to fear extinction training in animal models of emotional learning (Thyer et al., 1988; Foa & Kozak, 1986; Zarate & Agras, 1994).

Medications used to treat adult PTSD include antidepressants (e.g. Paroxetine, Mirtazapine, Amitriptyline), selective serotonin re-uptake inhibitors (e.g. Citalopram), serotonin–norepinephrine reuptake inhibitors (e.g. Fluoxetine), anticonvulsants (e.g. Carbamazepine, Divalproex, Topiramate), antipsychotics (e.g.

Risperidone, Clozapine), adrenoceptor agonists and antagonists (e.g. Clonidine, Prazosin), benzodiazepines (e.g. Alprazolam), monoamine oxidase inhibitors (e.g. Phenelzine), corticosteroids (e.g. Hydrocortisone) and NMDAR modulators (e.g. D-Cycloserine) (Frank et al., 1988; Davidson et al., 1990, 2006; Van der Kolk et al., 1994; Connor et al., 1999; Brady et al., 2000; Hageman et al., 2001; Martenyi et al., 2002; Peskind et al., 2003; Raskind et al., 2003, 2007; Onder et al., 2006; Berlin, 2007; Taylor et al., 2008; Schelling et al., 2001; Wingenfeld et al., 2013; Heresco-Levy et al., 2002; Litz et al., 2012; Philbert et al., 2013). Data exists for the efficacy of pharmacological interventions such as Clonidine (Perry, 1994; Harmon & Riggs, 1996), Carbamazepine (Loof et al., 1995), Risperidone (Horrigan, 1998) and Citalopram (Seedat et al., 2002) in child and adolescent PTSD. However, due to inadequate controlled studies, empirical evidence and in some instances safety data, medications are not recommended as treatment for child and adolescent PTSD. Instead, the first-line treatment for young PTSD sufferers is trauma-focused CBT with age-appropriate modifications (Goenjian et al., 1997; March et al., 1998; Cohen et al., 2000; King et al., 2000). Previous studies have also documented the efficacy of this treatment modality in children suffering from other anxiety disorders such as generalized anxiety disorder (GAD), seasonal affective disorder (SAD), and social phobia (SP) (Barrett et al., 1996; Kendall, 1994; Kendall et al., 1997; Silverman, et al., 1999; Pina et al., 2003).

Despite a substantial body of evidence supporting the efficacy of both treatments (Hofmann et al., 2008; Ravindran & Stein, 2010) – CBT and pharmacotherapy – many patients fail to respond adequately and relapses remain frequent (Holmes et al., 2003; Rescorla, 2004; Kessler et al., 2005; Kasper et al., 2010). During the past decade, researchers investigated the combination of psychotherapy and medication as treatment for PTSD i.e. applying these interventions simultaneously. However the effectiveness of

this approach also remains uncertain. In the present study we choose to focus on manipulating the glutamatergic system and more specifically the NMDAR. A few studies have reported positive results using NMDAR agonists in the treatment of PTSD (Ledgerwood et al., 2003; 2005; Walker et al., 2002; Yang & Lu, 2005; Litz et al., 2012; Philbert et al., 2013), while administration of NMDAR antagonists shortly after predator stress exposure was found to interfere with anxiety-related behaviour in rats (Adamec et al., 1999). Furthermore, a preliminary, retrospective study in a group of US military soldiers who had sustained thermal injuries during deployment, found that those treated with the NMDAR antagonist, ketamine, during hospitalization, had lower incidence of developing PTSD (McGhee et al. 2008). These findings therefore support the utility of NMDAR subunits as novel pharmacological targets for the treatment of PTSD.

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Chapter 3

The effects of D-cycloserine on fear conditioning and extinction

3.1 Introduction

Anxiety is similarly expressed in humans and animals (Ohl, 2003). Typical fear-associated responses observed in humans are displayed through behaviour in animals. These anxiety-like symptoms include escape, hyper-vigilance, avoidance and/or nonverbal vocalization (Palanza, 2001). Thus some parallels can be drawn between the human and animal brain. Animal models with useful criteria have been developed for studying the mechanisms causal to the formation and progression of anxiety disorders in humans (Van der Staay et al., 2009). Well established animal models such as fear conditioning and fear extinction have been developed to specifically study the pathogenesis and treatment of PTSD.

3.2 Fear conditioning

Pavlovian fear conditioning has progressively been recognized as a cross-species paradigm to study the learning and memory processes of fear. This model is defined by the pairing or unpairing of a neutral conditioned stimulus (CS) (e.g. a light or tone) with an aversive unconditioned stimulus (US) (e.g. a footshock), that yields a conditioned fear response (CR). The CR is elicited by the animal associating one or more stimuli with an aversive event. Rodent models utilizing aversive challenges (e.g. exposure to predators), intense stressors (e.g. electric shock) and situational reminders of trauma (e.g. time-dependent sensitization) have modeled the long-term behavioural effects, and autonomic and hormonal responses observed in the clinical PTSD setting (Adamec & Shallow, 1993; Pynoos et al., 1996; Liberzon et al., 1997; Cohen et al., 2003). Rats generally exhibit this

conditioned fear by freezing (Fench & Fanselow, 1999; Davis, 2000); a behaviour characterized by total lack of movement apart from respiration. Fear is displayed upon representation of either the context or the cue delivered in a different context. The abovementioned processes are respectively known as cued fear conditioning and contextual fear conditioning.

Cued and contextual fear conditionings are similar associative learning processes which differ on the grounds of a CS being added to the context. Furthermore, it was established that cued fear representations are formed in the basolateral amygdala (Phillips & LeDoux, 1992; Debiec et al., 2010), whereas contextual fear representations are processed in the hippocampal-cortical network (Phillips & LeDoux, 1992; Rudy & O'Reilly, 1999).

Cued fear conditioning involves the pairing of the CS and US. In cued fear conditioning the aversive stimulus (e.g. shock) is presented at the end of a neutral CS (cue e.g. tone) and thus the CS predicts the US i.e. involves pairing of the CS and US. This CS-US pairing results in a predominant conditional fear response to the tone as the context is in the background. Studies have shown that cue-specific or phasic fear is amygdala-dependent and characterizes anxiety disorders such as specific phobia (Grillon & Davis, 1997; Grillon, 2002).

Conversely, contextual fear conditioning involves the unpairing of the CS and US. This unpairing of the CS and US results in a predominant conditional fear response to the shock as the context is in the foreground. Contextual fear conditioning involves placement of the animal in a novel environment (e.g. shock box) and an US follows i.e. CS is absent or unpredictable of US. The rats first receive an electric shock and are subsequently tested

for their fear response. The rats learn that the context poses a threat in the form of an unsignalled shock. Studies have shown that contextual fear is hippocampal-dependent and has been found to characterize generalized anxiety. Contextual fear conditioning is considered the more rapid, robust form of the two associative learning procedures. Molecular studies of memory utilizing contextual fear conditioning have proposed that context-specific freezing is a major risk factor for anxiety (Grillon, 2002). Equally important were studies demonstrating that extinction within this context ameliorates conditioned fear (Graham & Milad, 2011).

Dysfunctional neuronal networks between the HC and other limbic areas have been implicated in anxiety states (Tsetsenis et al., 2007). For example, dysregulated hippocampal-dependent mechanisms, such as poor hippocampal-prefrontal cortex signalling has been shown to contribute to the putative fear learning abnormalities and sensitization reported in PTSD. Specifically, the modulation of contextual fear learning has been demonstrated to be dependent on dorsal hippocampal input. Previous fear extinction studies have shown this process to be particularly influenced by the specific contexts and to be facilitated by hippocampal-prefrontal cortex interactions (Sotres-Bayon et al., 2004; Ji and Maren, 2007). Pattwell et al. (2011) have further highlighted the inability of high trait anxious individuals to appropriately recruit prefrontal cortex regions in a context-selective manner, in order to facilitate the extinction of fear. Their finding prompts the question of whether the HC itself (i.e. impaired function) or dysregulated hippocampal-prefrontal cortex interactions contributes to the difficulty experienced by high trait anxious people when trying to facilitate fear extinction.

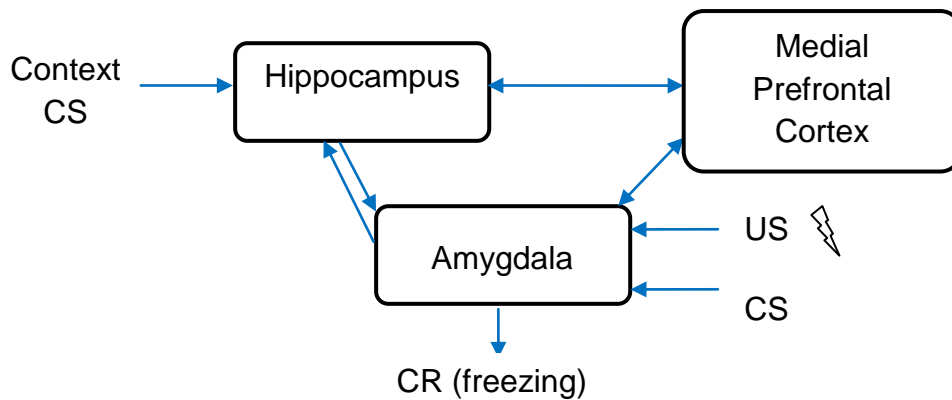


Figure 3.1: The fear conditioning circuit

3.3 Fear extinction

Fear extinction is defined by repetitive presentations of the CS in the absence of the US (Milad et al., 2006; Quirk et al., 2006; Rauch et al., 2006; Rothbaum & Davis, 2003). The CR associated with the CS begins to diminish over trials, as this response is no longer adaptive. In essence, subsequent to fear conditioning, the animal is trained that the CS no longer predicts the incidence of the aversive stimulus. Fear extinction thus represents a form of new learning whereby a decline in the magnitude and/or frequency of a CR is observed, following exposure to an extinction training protocol (Myers & Davis, 2002, 2007; Myers et al., 2010). The extinction protocol models relearning of the normal non-fear-motivated response to the cue or context, such as is utilized in clinical habituation/exposure-based therapies.

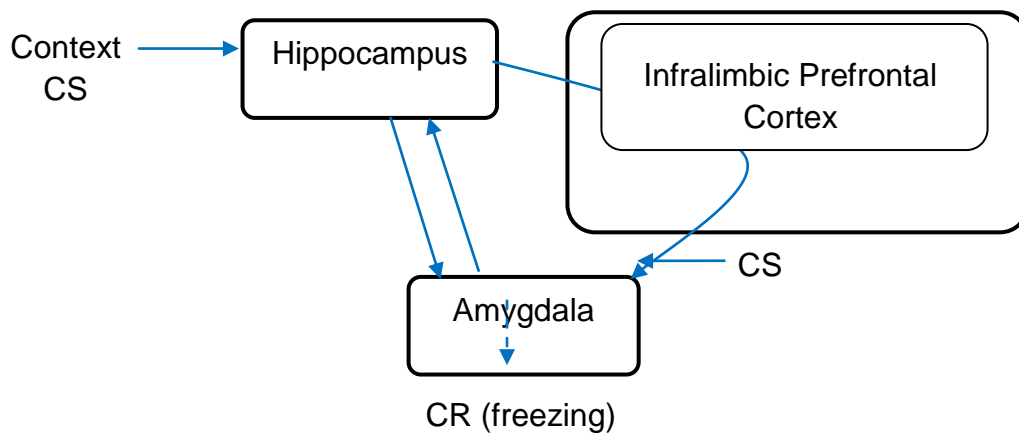


Figure 3.2: The fear extinction circuit

Procedurally analogous to fear extinction, EBT is a therapeutic process that entails repeatedly exposing patients to fear eliciting stimuli in a controlled environment (Zarate & Agras, 1994; Foa, 2000; Rothbaum & Davis, 2003). The patient cannot avoid the fearful object/situation and has to deal with it; realizing that nothing aversive is going to occur in this safe environment. The uncontrolled fears underlying the anxiety can thus be reduced over time. However, PTSD patients still experience frequent relapses as EBT does not readily transfer from the experimental set-up to actual anxiety provoking situations (Foa, 2000; van Minnen et al., 2002). Consequently, deficient fear extinction has been implicated in the development of PTSD. Even though the clinical relevance of extinction training is obvious, a better understanding of the underlying neural mechanisms involved, could improve PTSD treatments. It has been proposed that pharmacological drugs used in conjunction with EBT could aid the underlying processes of this therapy in treating anxiety disorders (Goldstein et al., 2001; Wrubel et al., 2007; Bredy et al., 2007; Bredy & Barad, 2008; Quirk & Mueller, 2008; Chang et al., 2009; Graham & Richardson, 2009 b; Milad et al., 2009a).

3.4 D-cycloserine, fear conditioning and extinction

Specific cognitive-affective processes (e.g. trauma sensitization, control of stereotypic movements, fear conditioning) are relevant to understanding different psychiatric disorders. Despite some of these processes being uniquely attributable to humans, a number of animal models have been developed to investigate the general stress response underlying most psychiatric disorders. Arguably, these animal models have contributed valuable insights, yet still lack an understanding of the particular mechanisms involved in the pathogenesis and treatment of particular disorders, such as PTSD.

To address this limitation, animal models of fear conditioning and extinction were developed. These processes were found to be relevant to the pathogenesis and treatment of numerous anxiety disorders (Gorman et al., 2000). These laboratory-based processes are particularly fundamental to studying PTSD as they have successfully modelled the physiological and behavioural responses seen in clinical PTSD settings (Rau et al., 2005; Siegmund & Wotjak, 2007; Mineka & Zinbarg, 2006).

Exposure-based therapy is employed as treatment for PTSD, yet has limitations in that not all patients have a significant response to this intervention and relapse is a common problem (Brown & Barlow, 1995; Foa, 2000; van Minnen et al., 2002; Hofmann & Smits, 2008). To maximize treatment response, the focus has been on investigating pharmacotherapies for use as adjuncts to EBT. The laboratory rat has proved to be a useful experimental model of traumatic experiences. Given that many aspects of neuronal and physiological development and measures of emotional reactivity in rats are predictive of events in humans (Blanchard et al, 2001).

Walker and colleagues (2002) were the first to demonstrate a role for DCS in enhancing fear extinction. The fear conditioning paradigm involved light and footshock pairings. Rats were infused with DCS, either systemically or directly into the amygdala, prior to extinction training. The fear response was measured by the fear potentiated startle. A retention test on the following day revealed DCS to enhance extinction in a dose-dependent manner with a concomitant decrease in the fear response. Following this report numerous studies have investigated this facilitation effect of DCS on extinction.

Despite administering lower doses, Ledgerwood et al. (2003) reported similar results. They found that either systemic DCS injections or intra-amygdalar DCS infusions facilitated the extinction of conditioned freezing. A follow-up study confirmed the positive modulation effects of DCS (Ledgerwood et al., 2004). DCS also seemed to block the occurrence of reinstatement-induced relapse (Ledgerwood et al., 2004). Interestingly, Parnas et al (2005) found that DCS still facilitated extinction when given up to 3 hours post-extinction training. In contrast, DCS failed to facilitate extinction when animals were pre-exposed to DCS. These authors specifically observed that 5 pre-exposures to DCS over a 10-day period abolished the enhancement effects. Their data suggested that only single-dose treatments have a positive effect on extinction. In support findings of Boje and colleagues (1993) showed that prolonged DCS exposure led to of NMDARs. This desensitization could explain the lack of positive effect of subsequent DCS treatment.

Other unexpected results were also observed. Briefly, Ledgerwood et al (2005) exposed fear conditioned rats to two different cues (i.e., a light and a tone) paired to an aversive noise. Twenty-four hours later, some rats underwent fear extinction training to the light and immediately received DCS or saline. The remaining rats were injected with DCS or saline without extinction training. All rats were tested the following day for fear responses,

by recording of freezing, to both cues. Results of the light experiment replicated earlier findings that DCS administration led to a reduction in fear of the light. Interestingly, rats injected with either DCS or saline in the absence of extinction training, showed no reduction in fear to the tone. Rats subjected to extinction training of the light combined with DCS, exhibited reduced fear of the tone. In other words, shocks administered post extinction training resulted in the conditioned fear returning to the control rats i.e. reinstatement. Experimental rats, exposed to DCS, sustained extinction i.e. displayed less reinstated fear. Accordingly, the combination of DCS and extinction training to one cue led to a reduction of fear to the second cue, previously paired with the same unconditioned stimulus. This reduction was termed 'generalised extinction'. While research on the facilitative effects of DCS on fear extinction was well underway, studies were yet to explain the molecular mechanisms underlying DCS-induced facilitation of fear extinction.

Previous studies have demonstrated a role for the signalling cascades MAPK and PI-3K in the extinction of conditioned fear (Lu et al., 2001; Lin et al., 2003). Considering these findings, Yang and Lu (2005) proposed that similar mechanisms may also be involved in DCS-induced facilitation of extinction. Their study was thus aimed at the amygdaloid NMDAR, MAPK and PI-3K cascades and their role in the extinction of conditioned fear. Yang and Lu (2005) reported that exposure to suboptimal extinction protocols modestly increased the activated forms of MAPK and protein kinase B (e.g. phospho-MAPK and phospho-protein kinase B) in the amygdala. Moreover, exposure to the same protocol in conjunction with DCS administration led to more robust increases in these kinases. Thus it was proposed that DCS activated NMDAR, resulting in Ca^{2+} influx which activated MAPK and PI-3K. These activated kinases translocated to the cell nucleus and ultimately promoted new protein synthesis and gene transcription (Yang & Lu, 2005). Given that

some molecular studies were conducted, the focus once again turned to the effects of DCS on post extinction relapse.

Woods & Bouton (2006) used the experimental model of renewal to test for the effects of DCS on the relapse of fear symptoms. Different doses of DCS were used in this study. Results indicated that DCS facilitated extinction of the conditioned emotional response at a high dose (e.g. 30 mg/kg). Their experiment also delivered a novel finding in that regardless of the drug dose, the extinguished fear was renewed upon the rats returning to the original context. This reactivation of an old fear memory was suggested to undergo a similar reconsolidation process as the original fear memory. Using the NMDAR-antagonist, MK801, Lee et al. (2006) found the reconsolidation of the reactivated fear to be blocked. Following this, Lee et al. (2006) hypothesized that when given prior to a memory reactivation task, DCS should potentiate the reconsolidation of fear memory by enhancing the conditioned fear. Their results demonstrated that DCS modulates both extinction and the reconsolidation of fear memory.

Previous animal studies postulated that extinction is an active learning process; inhibiting rather than erasing the original memory. Mao and colleagues tested this hypothesis in 2006. Their first finding was that an interlude between the conditioning and extinction of fear could determine whether a memory remained intact. Their second finding was that DCS facilitated the reversal of a fearful memory. These findings highlighted the importance of applying appropriate therapies within that critical time frame following exposure to a traumatic event.

Pre-exposure to DCS was suggested to prevent its standard effect. The findings of Werner-Seidler & Richardson (2007) corroborated that of Parnas et al. (2005) in this

regard. In addition pre-exposure to the antidepressant imipramine also prevented DCS from facilitating extinction. In other words, DCS might not enhance EBT in patients who concurrently are being treated with antidepressants.

The majority of research into the effects of DCS on extinction has primarily used visual cues as fear-eliciting stimuli. However, a strong association between odours and aversive events was demonstrated in both humans and animals. Thus Weber et al. (2007) was interested in the effects of DCS on extinction of odour cues. They showed that DCS may facilitate odour extinction. However, facilitation was dependent on within-session extinction occurring prior to DCS administration. Furthermore, they found that DCS facilitated long-term extinction of these fear responses. This finding corroborated with that of Lee et al (2006), in that DCS facilitated memory reconsolidation. As noted in the above studies (also see Table 3.1 below), systemic administration either before (Walker et al., 2002; Ledgerwood et al., 2003, 2005; Yang & Lu, 2005; Mao et al., 2006; Woods & Bouton, 2006; Weber et al., 2007) or after (Ledgerwood et al., 2003, 2004, 2005; Parnas et al., 2005; Weber et al., 2007; Werner-Seidler & Richardson, 2007) extinction training facilitates extinction. Similarly, DCS was shown to facilitate extinction when directly infused into the basolateral amygdala, either before (Walker et al., 2002; Lee et al., 2006; Mao et al., 2006, 2008) or after (Ledgerwood et al., 2003) extinction training (Table 3.1).

Mao and colleagues (2008) confirmed that intra-amygdalar DCS infusion, prior to extinction training, enhanced reduction of the startle potentiation. Extinction was facilitated without altering the baseline acoustic startle and sensorimotor responses (Walker et al., 2002; Ledgerwood et al., 2003). In 2008, Bouton and colleagues re-analyzed previously reported data from their null result experiments (Woods & Bouton, 2006). They found that DCS facilitated extinction learning when combined with a moderate number of extinction

trials. This finding was consistent with the Lee et al (2006) study; showing that the number of extinction trials is essential to the efficacy of DCS on extinction. Their 2008 study also affirmed the results of their 2006 study (Wood & Bouton, 2006; Woods & Bouton, 2008).

Despite its positive benefits DCS did not change the context-dependence of extinction learning. Langton and Richardson (2008) confirmed previous findings of the facilitatory effects of DCS on extinction without any effect on the re-extinction of conditioned fear. McCallum and colleagues (2010) investigated the effects of DCS on adolescent rats and found that systemic DCS injections (dose of 1.0 mg/kg), given post extinction, impaired extinction retention in this population during an extinction test given 24 hours later. By increasing the DCS dosage to 15mg/kg, extinction retention was shown to improve and the adolescent rats completely recovered from their previously exhibited fear (McCallum et al., 2010).

Table 3.1: Studies examining the effect of DCS on extinction in fear conditioned rats

DCS administration dosage	Locus of DCS administration	Effect of DCS administration	Reference
Pre-extinction 2.5 – 30 mg/kg	Systemic	Facilitated extinction	Walker et al., 2002, Polese et al., 2002; Ledgerwood et al., 2003; Bouton et al., 2008; Yamamoto et al., 2008; Lin et al., 2009
Post-extinction 2.5 – 30 mg/kg	Systemic	Facilitated extinction	Ledgerwood et al., 2003; Ledgerwood et al., 2004; Weber et al., 2007; Werner-Seidler & Richardson, 2007
Post-extinction 15 mg/kg	Systemic	Generalized extinction	Ledgerwood et al., 2005
Pre-extinction 10 µg/side	Basolateral amygdala	Facilitated extinction	Walker et al., 2002; Ledgerwood et al., 2003; Mao et al., 2006, 2008
Pre-extinction 10 µg/side	Basolateral amygdala	Increased GluR1 expression	Mao et al., 2006
Pre-extinction 15 mg/kg	Systemic	Facilitated 1 st but not 2 nd extinction	Langton & Richardson, 2008

3.5 D-cycloserine: concluding remarks

This chapter highlights the potential contribution of animal models in understanding the fundamental mechanisms underlying anxiety disorders as well as in pharmacological drug development for these disorders (Lister, 1990; Martin, 1998). All reported rat studies demonstrated enhanced fear extinction effects when DCS was administered immediately before or shortly after extinction training.

The primary finding of the studies reviewed here was that DCS facilitates extinction of conditioned fear. Additional facilitative effects were found on: consolidation of conditioned fear (Ledgerwood et al., 2003), consolidation of fear memories (Ledgerwood et al., 2003;

Lee et al., 2006), and generalization of conditioned fear (Ledgerwood et al., 2005). Furthermore it was shown that chronic DCS exposure prior to fear conditioning decreases the effectiveness of DCS on extinction (Parnas et al., 2005; Lee et al., 2006), by appearing to temporarily change the glycine/NMDAR complex function. However, it was noted that a drug free interval of 28 days interposed between pre-exposure and fear conditioning, restored the augmentation effects of DCS. This finding supports previous work by Quartermain et al., (1994), who reported that 15 days of continuous DCS treatment resulted in significantly poorer spatial memory retention in mice compared to mice who received single injections. It was suggested that chronic NMDA activation caused receptor desensitization; implicating this glutamatergic system in the consolidation and maintenance of fear extinction, and thus the pathophysiology of PTSD. Conversely, this finding of Parnas et al. (2005) is in contrast to that of studies of DCS initially conducted (Walker et al., 2002; Ledgerwood et al., 2003). A study performed by Yamamoto et al. (2007), demonstrated that post systemic administration of DCS enhanced fear extinction and proposed NMDAR desensitization (Parnas et al., 2005) may not have happened yet. However, the differences in the experimental procedures of these studies should be considered as they may have led to the observed discrepancy. This discrepancy also pointed out the importance of considering the amount of DCS exposures and intervals separating these exposures.

DCS was also found to reduce spontaneous recovery (Walker et al., 2002; Ledgerwood et al. 2003; Lee et al., 2006) and reinstatement (Ledgerwood et al., 2004) but not renewal (Woods & Bouton, 2006) or rapid reacquisition (Walker et al., 2002; Ledgerwood et al., 2005) of conditioned fear. These findings may be explained in light of enhanced contextual inhibition resulting from DCS promoting extinction learning to the background context.

This review also demonstrated that an administration schedule is crucial for the efficacy of DCS. Considering the half life of the drug; administration has to be within four hours of extinction training. In some instances a failure to exhibit reinstatement has also been observed when doses were administered post extinction; these studies also highlighted the importance of the context within which extinction took place. A novel study investigating the adolescent period demonstrated that by doubling the extinction training and systemic DCS administration, alleviation of impaired extinction was possible (McCallum et al., 2010). More studies are needed in this regard as early developmental trauma has been shown to be fundamental in understanding PTSD (Heim & Nemeroff, 2001; Coplan et al., 1996).

It can be concluded that DCS is a promising adjunct to fear extinction, but further research remains to be conducted to establish the complete behavioural consequences of the drug specifically in the pre-clinical setting and when administered during the adolescent period.

3.6 Importance of studying PTSD in the adolescent period

Early developmental trauma has been shown to alter the risk for behavioural abnormalities and psychiatric disorders later in life (Heim & Nemeroff, 2001; Kendler et al., 1992). Thus the developmental stage of the individual at the time of trauma exposure is of importance. Alterations in brain function at particular time periods are critical in the development of human stress-related psychopathologies (Bogerts, 1989; Petersen et al., 1996; Spear, 2000) and the development of anxiety-like disorders in animals (Heim et al., 2004; Maercker et al., 2004; Nemeroff, 2004). Studies specifically reported a noticeable peak in the development of anxiety and other affective disorders during the adolescent period (Kessler et al., 2005; Kim-Cohen et al., 2003).

Adolescence is a critical developmental period, characterized by the continuous organization of neural circuits regulating emotional and learning processes (Spear, 2000; Casey et al., 2008). These extensive neural changes were observed in the prefrontal cortex, (Genazzani et al., 1997; Insel et al., 1990; Jernigan et al., 1991; Kalsbeek et al., 1988; Sowell et al., 1999; Van Eden et al., 1990), HC (Andersen & Teicher, 2004; Insel et al., 1990; Michelson & Lothman, 1989; Nurse & Lacaille, 1999) and amygdala (Kellogg, 1998; Terasawa & Timiras, 1968) of adolescent rats and found to enhance stress perception and response. Subsequent preclinical and clinical studies documented the long term effects of stress exposure during the adolescent period; confirming that such stress-related events manifest as psychopathologies later in life (Spear, 2000, 2004; Heim & Nemeroff, 2001; Heim et al., 2004; Maercker et al., 2004; Nemeroff, 2004).

A recent mouse model investigated the effects of hippocampal-dependent contextual fear conditioning and amygdala-dependent cued fear conditioning on fear learning and memory processes in the period into and out of adolescence (Pattwell et al., 2011). Previous research indicated that contextual fear learning is present at a very early age i.e. in rats younger than PND 29 (Rudy, 1993; Moriceau & Sullivan, 2006). This finding prompted Pattwell and colleagues (2011) to investigate the absence or suppression of contextual fear in the early-adolescent period. Three different cohorts pertaining to adolescence were used, namely postnatal day (PND) 29, 39, and 49 which correlate to early, mid, and late adolescence, respectively (Spear, 2000; Hefner & Holmes, 2007; Adriani et al., 2004). An adult cohort, PND 70, was used for baseline comparisons. Furthermore the authors decided to study their adolescent cohorts at two time points namely, immediately before (PND 23–27) and immediately after (PND 31–49) the adolescent period. Patwell et al. (2011) reported that early pre-adolescent (PND 23-27) fear conditioned mice exhibited complete contextual fear identical to that exhibited by the adult cohort. The contextual fear

was subdued upon transition into adolescence (PND 29), whereas proceeding out of the adolescent period (PND 39) resulted in a re-emergence of the contextual fear.

Pattwell et al. (2011) thus suggested a period of suppression prior to and following adolescence (PND 29). During this interlude the context in which the fear conditioning was presented, was relayed as a cue and constructed as a fear memory. This period of suppression correlated to Fanselow's theory which states that "the contextual representation of the conditioning environment must be relayed to the affective system as a fear-signifying cue after, and only after, such a representation has been constructed by the cognitive system" (Fanselow, 1994). Furthermore, the period of suppression of contextual fear, as noted in pre-adolescent and adult mice (Pattwell et al., 2011), was consistent with previous findings (Chen et al., 2005; Rainecki et al., 2010). Basically the interlude implied that the context in which the fear conditioning was presented was unable to elicit a fear response until the mice reached adulthood. This brief suppression can be viewed as an evolutionary, compensatory/survival mechanism. Increased contextual fear would be maladaptive to exploratory behaviour in the pre-adolescent rat since this time period is when rodents explore their surrounds, become independent and ultimately leave the nest (Spear & Brake, 1983). This study poses an interesting concept in that fear memories formed during this suppression period remain intact and may be retrieved at a later stage, i.e. adulthood. Replication of this study is therefore important to better elucidate the inherent mechanisms involved in suppressing unwanted fear memories.

3.7 Summary

Defined as a fear-based disorder, PTSD develops upon exposure to an extremely threatening event (e.g. war combat) and is associated with a conditioned fear response.

The conditioned fear response is brought about by the traumatic exposure; eliciting the arousal symptoms of PTSD. Efforts to escape the feared stimuli and subsequent emotional response are recognized as the avoidance symptoms of PTSD. Exposure-based therapy utilizes exposure to the feared stimuli in the absence of negative consequences with the aim to extinguish the conditioned fear response. Laboratory based models of fear conditioning and extinction have been developed to examine behaviours provoked by stimuli associated with an aversive event (e.g. electric footshock).

Animal models of fear conditioning and extinction elicit robust changes in behaviour which can be easily measured and quantified. These laboratory-based processes are fundamental to studying PTSD as they have proven successful in modelling the physiological and behavioural responses seen in clinical PTSD settings (Mineka & Zinbarg, 2006). Although fear conditioning studies focusing on adolescents are in their infancy, more researchers have noted the significance of this critical developmental period in order to timeously identify and treat trauma-related psychopathologies, such as PTSD. DCS was shown to improve EBT and fear extinction outcomes alike. These findings offer a potential advantage for DCS over other pharmacotherapy augmentation strategies which have been associated with a greater risk of relapse after discontinuation (Marks et al., 1993).

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CHAPTER 4

Methodology

4.1 Materials and Methods

4.1.1 Animals

Adolescent (PND 33) and Adult (PND 60) male Sprague-Dawley rats (200 – 350 g) were used in this experiment. Rats were used instead of mice, as they demonstrate more complex social behaviours which allows for finer behavioural assessments. In addition, rats are larger in size than mice, making rat brain tissue sampling easier. The university's animal facility has also found it easier to acquire and breed the Sprague-Dawley rat strain, which was found to model anxiety disorders better. Therefore, our Neuroscience laboratory has designed and established behavioural tests to measure anxiety-like symptoms in this specific rat strain.

Animals were housed at the Central Research Animal Facility (AAALAC accredited) of the University of Stellenbosch in a temperature-controlled (22°C, 55% humidity) colony room and maintained on a 12-h light/dark cycle (lights on at 6:00 am). The colony room was separate from where fear conditioning procedures, DCS exposures or dissections took place. Cages were provided with sterilized corn cob as cage litter, with rat chow (food) and water available *ad libitum*. Rats were housed 2–4 animals per cage, which allowed for social interaction i.e. playing and grooming of companions. However, post-surgery 1 rat was housed per cage for recovery purposes as well as minimizing danger that a companion might groom and nibble at the wound.

Housing conditions and experimental protocols followed in this study were approved by the Committee for Experimental Animal Research of the University of Stellenbosch (Project Number: P08/05/007), and were in accordance with the University's Guide for Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

4.1.2 Drugs

D-cycloserine was procured from Aspen Pharmacare (South Africa). The anaesthetics Ketamine hydrochloride (Anaket-V), Medetomidine hydrochloride (Domitor) and Antisedan, the antibiotic Trimethoprim sulfadiazine and analgesic Flunixin Meglumine were all purchased from Lakato Vets (South Africa).

4.2 Experimental Design

In total 300 rats were used in the study. The rats were divided into two main groups namely, adolescent and adult groups. The two main groups were further subdivided into 5 subgroups each, yielding a total of 10 groups. Subdivided groups were as follow (n=30/adolescent group; n=30/adult group):

1. Naïve group (N): animals not subjected to fear conditioning and not receiving intrahippocampal injections (to provide baseline values).
2. Control Saline group (C Sal): animals not subjected to fear conditioning and receiving intrahippocampal saline injections.
3. Fear-conditioned Saline group (Fear Sal): animals subjected to fear conditioning and receiving intrahippocampal saline injections.

4. Control D-cycloserine group (C DCS): animals not subjected to fear conditioning and receiving intrahippocampal DCS injections.
5. Fear-conditioned D-cycloserine group (Fear DCS): animals subjected to fear conditioning and receiving intrahippocampal DCS injections.

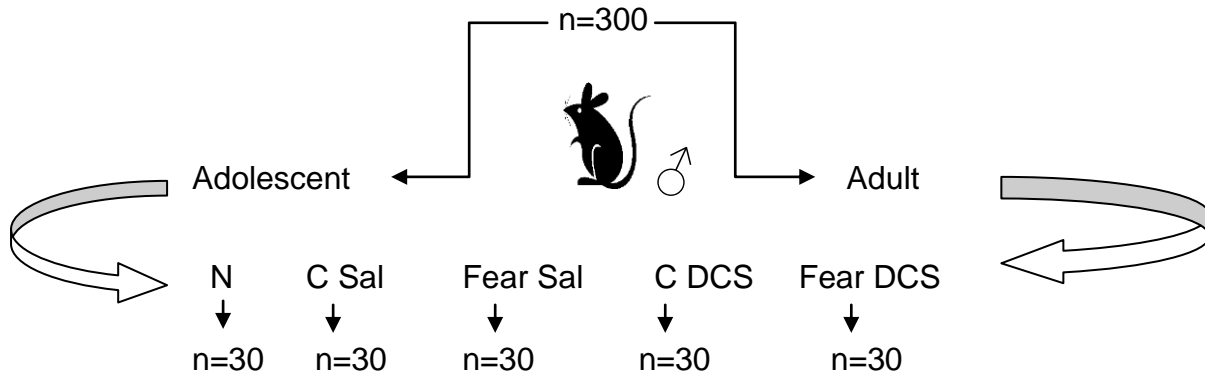


Figure 4.1: Experimental design overview flow diagram. Total of 300 Sprague-Dawley rats divided into two main age groups; adolescents and adults. Subdivision of the age groups into 5 experimental groups. N – naïve, C – control, Sal – saline, Fear – fear conditioned, DCS – D-cycloserine, n – number of animals/group.

4.3 Posttraumatic stress disorder animal model

As previously mentioned, PTSD can be categorized under two memory processes, namely those relating to the memory of the trauma (i.e. associative memories: avoidance, re-experiencing) and those which lack such an association (i.e. non-associative memories or sensitisation: hyperarousal, blunted emotionality, social withdrawal). For this reason we chose a PTSD animal model (Siegmund and Wotjak, 2007) which incorporates both memory processes in the development and maintenance of PTSD.

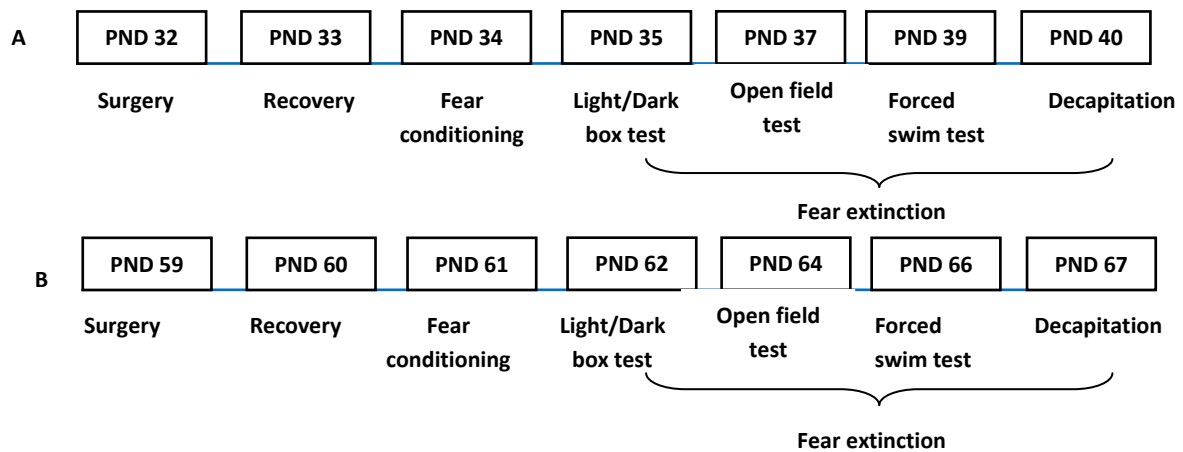


Figure 4.2: (A) Experimental timeline of the current PTSD model demonstrating the behavioural procedures conducted on adolescent (PND 33) animals. (B) Experimental timeline of the current PTSD model demonstrating the behavioural procedures conducted on adult (PND 60) animals. Fear conditioning consisted of a series of 10 single electric footshocks applied in one minute. Fear extinction involved the presentation of a 1 minute tone in a neutral chamber. PND – post natal day

4.3.1 Fear conditioning and fear extinction

The proposed fear conditioning paradigm was based on the mouse model of Siegmund and Wotjak (2007). The mouse model was modified to suit the behavioural assessment of our animals. Firstly male Spague-Dawley rats were used instead of C57BL/6N [B6N] and C57BL/6JOla [B6JOla] mice as in the Siegmund & Wotjak study (2007). Secondly, the intensity and duration of the electric footshock was increased in order to elicit a valid fear conditioned response in the rats. This consisted of 10 repetitive footshocks (each lasting one second) administered over a one minute period (rat model) versus the two second footshock (mouse model). Thirdly, a bigger open field was utilized as the apparatus used in the mice model would be too small to prompt anxiety-like symptoms in the bigger rodent species (rats).

Fear conditioning commenced on PND 34 and 61, for adolescent and adult rats respectively. A Perspex chamber (20X20 cm) with a metal grid floor, connected to an electric current supplier (Model HFCG-R, from Lafayette instrument, www.lafayetteneuroscience.com) was used to administer a series of single electric foot shocks (shock intensity, 1.5 mA; shock duration, 1 s; 10 repetitive shocks over a period of 1 minute). This served as the aversive encounter (the unconditioned stimulus). In order to facilitate the context association to aversive stimuli, rats remained in the chamber for an additional 60 seconds after receiving the footshock stimulation and were then returned to their home cage. Control animals were also placed in the shock chamber, but did not receive any footshocks. To test for conditioned fear (associative fear), the animals were re-exposed to the shock chamber for three minutes, without further shock application. Memory retention was evaluated by analyzing their contextual freezing behaviour. They were then returned to their home cages. Compartments and the floor of the shock box were thoroughly cleaned with soap water between trials so as to remove any associative cues (e.g. odours, faecal boli) which might influence the following test subject and become a confounding factor.

The shock chamber was accompanied by a tone box/apparatus that was calibrated to emit a sound of frequency 9kHz and 80 dB. In order to test for sensitized fear (non-associative fear), the animals were placed in a neutral test chamber and exposed to the same tone (80 dB, 9 kHz) after three minutes. After the tone presentation, the animals remained in the neutral chamber for another 60 seconds, before being returned to their home cages. Fear extinction was conducted over a period of 6 consecutive days. The repetitive exposure of the animals to the contextual cue (initial shock chamber) in the absence of footshock, served as the fear extinction protocol. DCS infusions were done in conjunction with the fear extinction procedures.

The core features of PTSD were subsequently recorded in the following manner:

- 1) the freezing response when re-exposed to the shock context as a measure of conditioned fear (associative fear memory);
- 2) the freezing response to a neutral tone in a novel context, as a measure of the sensitized fear (non-associative fear memory); and
- 3) the freezing response during repetitive exposure to the shock context when no shock is given, as a measure of fear extinction learning.

Freezing behaviour was defined as the immobility of the animal except for respiration movements (Holahan and White, 2002).

4.3.2 Behavioural Assessment

Behavioural procedures were conducted during PND 35-40 (adolescent period) and PND 62-67 (adulthood) during the light phase of the day/night cycle between 10:00 A.M. and 13:00 P.M, to minimize circadian influences. Behaviour was recorded with a mounted video camera and analyzed using Noldus Ethovision software (Wageningen, The Netherlands). To test for various signs of emotional distress, the following tests were employed respectively: light/dark avoidance test, open field test and the forced swim test.

4.3.2.1 Light/Dark avoidance test

The light/dark paradigm (Crawley & Goodwin, 1980) is based on the innate aversion of rats to brightly lit areas in addition to their spontaneous exploratory behaviour. This test creates a natural conflict situation; whereby the rat has a natural desire to explore the

novel environment yet at the same time seeks shelter/avoid the unfamiliar. Therefore the light/dark avoidance test is useful in indicating heightened anxiety-like behaviour in rats and has been used in this regard before (Cacciaglia et al., 2013; Adamec et al., 2010).

The test arena consists of an illuminated and dark compartment of equal size, which is connected by a doorway. Rats were placed in the lit area and habituation time of 5 minutes was allowed in order to eliminate the stress of a new environment. The behaviour of the animals was recorded during the subsequent 5 minutes. After the 10 minute period the rats were returned to their home cages. End-parameters such as relative time spent in the dark compartment were recorded.

The floor of the arena was cleaned with soap water in between each trial.

4.3.2.2 Open field test

The open field test is based on conflicting innate tendencies of avoidance of bright light and open spaces and of exploring a novel environment. The open field test can also be used for general assessment of animal basal locomotor activity and exploration. This test is therefore useful to assess levels of anxiety, hyperarousal and hyperactivity in rats (Miao et al., 2014; Qiu et al., 2013).

The 1mX1m square box is divided into an inner and outer zone. Animals were placed into the bottom right quadrant of the board and behaviour recorded with the video camera for a

period of 10 minutes. Parameters measured included: time spent in outer and inner zones, and the total distance travelled.

The box was cleaned with soap water, in between each trial.

4.3.2.3 Forced swim test

The forced swim test is commonly used to measure depression-like behaviours in rats and therefore provides insights into the level of helplessness in rodents (Ji et al., 2014).

The test comprises the use of perspex cylinders (18 cm diameter), filled to a height of 15 cm with ambient (25 °C) water. Rats were gently placed into the water for a time period of 10 minutes after which they were towel dried and returned to their home cages. The water was exchanged after each experimental run (Avital et al., 2001; Hall et al., 2001). End-parameters such as immobility (absence of movement except for those necessary to keep head above the water), swimming (forward motion through the water) and struggling were scored.

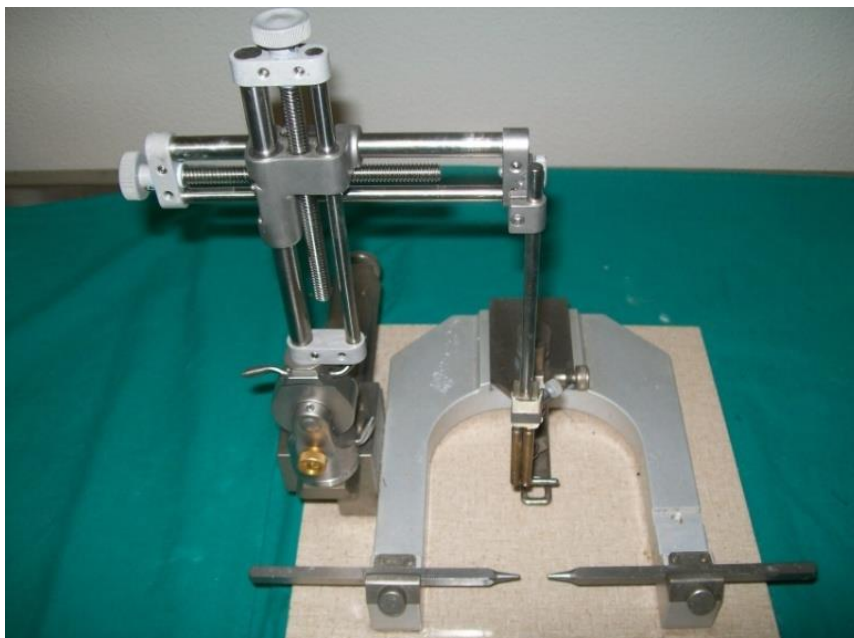
4.4. Infusion of D-cycloserine into the dorsal hippocampus

4.4.1 Preparation of cranium

All surgical instruments were sterilized by means of autoclaving and non-touch, aseptic surgery was performed. All surgery procedures were carried out under anaesthesia on PND 32 (adolescents) and PND 59 (adults). Rats were manually restrained for a short

period while a combination of ketamine hydrochloride (70 mg/kg) and medetomidine hydrochloride (0.5 mg/kg) was administered intraperitoneally. Once anaesthetized, the head of the animal was shaved and the area cleaned with 70% Ethanol (Kimix, South Africa). The rat was mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) attached to a thermostatically controlled heat pad (REX C10, $19.0 \pm 99.9^{\circ}\text{C}$, Electronic Services, University of Stellenbosch) which maintained the body temperature. The cranium was levelled in the horizontal plane using the ear bars and incisor bar. An incision of approximately one centimetre was made into the skin covering the cranium. The connective membranous tissue overlying the skull was removed, using curved surgical forceps and the exposed area sterilized with Iodine (Purdue Pharmaceuticals, South Africa).

Photograph 4.1: Stereotaxic apparatus



4.4.2 Determination of stereotaxis coordinates

The brain area coordinates for placement of cannulae were deduced from “The rat brain in stereotaxic coordinates 2nd edition” (Paxinos & Watson, 1986). A three-dimensional map of the brain (stereotaxic atlas: Paxinos & Watson, 1986) along with the vernier scales on the apparatus, guided the placement of the cannulae for the intrahippocampal infusions. Stereotaxic coordinates were read relative to Bregma, in order to cancel out the individual variability observed in skull thickness. Bregma is the point on top of the skull where the coronal and sagittal sutures meet.

To verify that the infusion site essentially corresponds to the DHC, the brains of two adolescent (200-250g) and two adult rats (300-350g) were infused with 10 μ l India ink at the predetermined coordinates. Following the ink-infusion, the rats were decapitated, their brains removed and stored in 10% formalin for at least 72 hours. Thereafter the tissue was sliced into 40 μ m coronal sections, with a cryostat. The brain slices were mounted onto clear glass slides and verified for accurate cannula placement and spread of the dye in the DHC as previously described (Quevedo et al., 1999, 2004). The coordinates corresponding to the DHC were anteroposterior (AP), - 4.5 mm from bregma, mediolateral (ML), \pm 2.5 mm from bregma, dorsoventral (DV), - 3.5 mm from dura.

4.4.3 Implantation of guide cannulae

Two burr holes were drilled into the skull at the precise coordinates (- 4.5 mm AP, \pm 2.5 mm ML, - 3.5 mm DV) that corresponded to the target area i.e. left and right DHC as obtained from the atlas of Paxinos and Watson (1986). Intracranial indwelling guide cannulae (11 mm, 22 gauge, Plastics One Inc, USA) were carefully lowered down into the

bilateral holes for the administration of either saline or DCS at a later stage. The cannulae were secured with dental cement (Department of Dentistry, University of Stellenbosch) and four stainless steel skull screws were fitted into the skull which further anchored the entire headpiece. Stainless steel stylets (36 mm, 30 gauge, Plastics One Inc, USA) were placed into the guide cannulae to prevent particles from entering the brain and keeping it patent throughout the experiment.

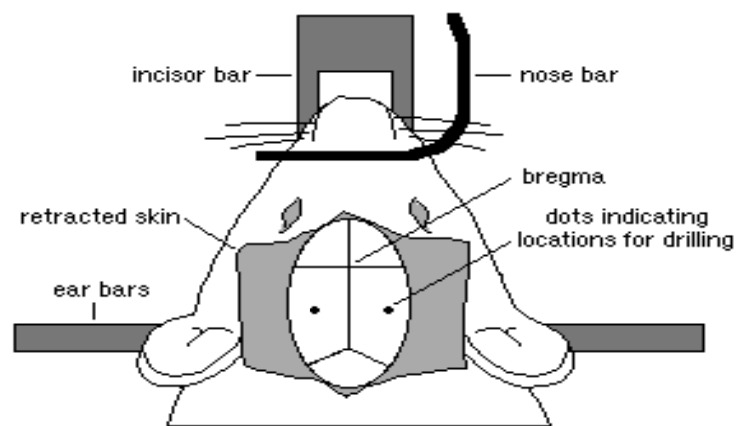


Figure 4.3: A diagram representation of a rat's head in the stereotaxic apparatus

4.4.4 Post-operative care

To ensure full recovery, animals received post-operative antibiotics (Trimethoprim sulfadiazine 30 mg/kg once daily for 5 days) and analgesia (Flunixin meglumine 2.5 mg/kg once daily for 3 days). The rats were allowed to rest for at least one day after surgery, PND 33 (adolescents) and PND 60 (adults). To assess the rats' wellbeing they were examined twice daily, in their cages, for signs of distress. These signs included checking the quality of the animals' coat (pilorection), their food and water intake as well as general locomotor activity. It was not anticipated that the experimental procedures and/or the

study drug (DCS) would induce illness. However, provisions were made that if signs of illness were evident in an animal, a faculty veterinarian would be consulted.

4.4.5 Bilateral DCS/Saline infusions

As DCS is unstable in solution (Yang & Lu, 2005), it was prepared fresh daily, immediately before administration. DCS was dissolved in physiological (0.9%) saline. DCS-infusions were administered for 6 consecutive days, 30 minutes prior to fear extinction sessions at a dose of 10µg per rat (Walker et al., 2002). Bilateral DHC infusions of the drug/vehicle took place on PND 35-40 (adolescents) and PND 62-67 (adults) by using a Hamilton syringe with a 22-gauge needle. A total volume of 0.5 µl of DCS was infused into each of the hippocampi. Control animals received equivalent amounts of the vehicle (0.9% saline). The needle remained in place for 2 minutes to allow the drug/vehicle to diffuse away from the injector. An air bubble was aspirated prior to infusion of DCS/saline, to allow for complete evacuation of the DCS solution. The animals were weighed each day so as to determine the amount of DCS/saline to be administered and at the same time also familiarizing them to being handled. To further minimize additional stress, the animals received DCS-infusions during the light phase of the light/dark cycle, to ensure their circadian rhythms remained undisturbed.

4.5 Neurochemical Analyses

Animals were decapitated upon completion of the experiments on PND 40 (adolescent) and PND 67 (adulthood) respectively. The DHC was dissected and appropriately stored for later biochemical analysis. Enzyme-Linked Immuno-Sorbent Assay (ELISA) kits were used for measuring NMDAR, BDNF and nNOS concentrations, and isobaric tag for relative

and absolute quantitation (iTRAQ) mass spectrometric methodology was used to measure protein expression levels.

4.5.1 Measurement of NMDA receptor levels

The DHC was used for the determination of NMDAR levels, as these receptors appear to have a critical role in extinction of contextual fear conditioning (Walker et al., 2002; Ledgerwood et al., 2003, 2004, 2005). NMDA-receptor subunit 1 (NMDAR1) levels were measured with a commercially available ELISA kit (MyBioSource, Inc. San Diego, USA). This subunit was chosen as it represents the binding site for the agonist, DCS.

Tissue samples were rinsed in ice-cold PBS (0.02 mol/l, pH 7.0-7.2) to remove excess blood thoroughly and then weighed before homogenization. The resulting homogenate was sonicated to further lyse the cell membranes (10 seconds at 20 Hz) and then centrifuged at 5 000 rpm at 4 °C for 15 minutes. The supernatants were aliquoted and stored at -20 °C. All determinations were performed in duplicate. Results are expressed in pg/mg wet weight.

4.5.2 Measurement of BDNF concentration levels

Dorsal hippocampus tissue samples were weighed and suspended in 400 µl lysis buffer (137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 % Nonidet P-40 (NP 40), 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 µg/ml leupeptin and 0.5 mM sodium vanadate). The samples were then sonicated for 40 s in the lysis buffer, vortexed and then centrifuged at 14 000 rpm at 4 °C for 20 minutes. The supernatants were

aliquoted into test tubes for the determination of BDNF concentrations using the Emax ImmunoAssay system (Promega, Madison, USA). ELISA kits were completed following the manufacturer's instructions. Determinations were made in duplicate. Results are expressed in pg/mg wet weight.

4.5.3 Measurement of nNOS concentration levels

Dorsal hippocampus tissue samples were weighed and prepared in the same manner as the BDNF aliquots i.e. 400 µl lysis buffer, centrifuged at 14 000 rpm 4 °C for 20 min. nNOS concentrations were determined using a commercially available ELISA kit (Alpco Diagnostics, Salem, USA). Determinations were made in duplicate. Results are expressed in pg/mg wet weight.

4.5.4 Measurement of protein changes (done in conjunction with the Center for Proteomic and Genomic Research, University of Cape Town)

4.5.4.1 Protein digestion and labelling

The DHC tissue was trimmed to approximately 10mg. Protein extraction was initiated by adding 250 µl (25 volumes) of the extraction buffer (100 mM TEAB (Triethylammonium bicarbonate; Sigma), 100mM NaCl (Riedel-de-Haën), 10 mM EDTA (Ethylenediaminetetra acetic acid (Fluka), 0.5% OGP (Octyl-glucoopyranoside, Sigma), 1% DOC (Sodium deoxycholate, Sigma), 5 mM TCEP, (Triscarboxyethyl-phosphine; Fluka), 0.4% Protease inhibitor cocktail) to the tissue. The tissue was disrupted using a Dounce tissue grinder with pestle number B (0.0005 -0.0025 inch clearance). The samples were subjected to 3 cycles of sonication for 5 minutes on ice followed by vortex mixing; the samples were subsequently centrifuged at 22 000 x g for 20 minutes. The supernatants were collected

and precipitated overnight at -20 °C using 4 volumes acetone. The samples were then centrifuged at 12 000 x g for 10 minutes and the supernatant was discarded. The pellet was re-suspended in 100mM TEAB, 4M Guanidine Hydrochloride (Sigma), 0.5% OGP and sonicated on ice until dissolved. Protein concentration was determined by measuring the absorbance of the solution at A280 nm using a Nanodrop ND 1000 spectrophotometer. The protein concentration of all the samples was normalized by adjusting to the sample with lowest concentration. Prior to digest α -casein was introduced at 6pmol to serve as control for digest and labelling steps. Sample volumes were further adjusted to 10 μ l with 50mM TEAB. In order to reduce the various proteins in the sample 1 μ l 100mM triscarboxyethyl phosphine (TCEP) in 50 mM TEAB was added to the protein suspension. After this step the samples were incubated for 1 hour at 60°C. After reduction the samples were cooled to room temperature and all cysteine residues alkylated by adding 1 μ l methyl-methanethiosulfonate (MMTS, Sigma, final concentration 5mM) to the protein suspension and incubating it for 30minutes. The samples were then diluted with 50mM TEAB to 45 μ l before 5 μ l trypsin (Promega) solution (1mg/ml) was added. The samples were finally incubated overnight (18 hours) at 37°C.

4.5.4.2 iTRAQ labeling

The digest was evaluated using reverse phase chromatography prior to labelling. Sample volumes of each of the protein digest were reduced to 10 μ l in a roto-evaporator (LabConco). To the concentrated samples 10 μ l of 600mM TEAB were added to a final concentration of 300mM and the pH confirmed as being greater than pH 7.5. The iTRAQ reagents (AB Sciex) were prepared as per manufacturer's instructions and the vials containing the labels, centrifuged. The contents of the vials were added to the respective samples. After the samples were vortex and centrifuged all the tubes were incubated at

room temperature for two hours. After two hours 1µl aliquots were removed from each of the samples and combined for desalting. The samples were double diluted with loading solvent (2% ACN/water, 0.15% TFA (acetonitrile, Burdick and Jackson; trifluoroacetic acid, Sigma). The combined sample was desalted using C18 ZipTips (Millipore) as per manufacturer's instructions. The desalted sample was mixed with 10mg/ml α -cyano-4-hydroxy-cinnamic acid (CHCA, Sigma) in 50% ACN/water; 0.1% TFA, and spotted onto a matrix-assisted laser desorption ionization (MALDI) target plate.

4.5.4.3 First dimension chromatography

4.5.4.3.1 Column preparation

Hydrophilic interaction chromatography was performed using anion exchange (SAX; quaternary amine; Supelco). One hundred milligram (100mg) of resin was washed twice with 1ml 100% acetonitrile (ACN, Burdick and Jackson). The washed resin was degassed in a desiccator for 30 minutes prior to packing the columns. The columns were packed in 1ml polyethylene filtration tubes (Supelco). The columns were $\frac{3}{4}$ filled with ACN and the media in ACN added. The added media were allowed to settle under gravity. Once the chromatographic media had settled a frit was inserted and the columns equilibrated with 60% ACN/20 mM TEAB adjusted to pH6.8 with HCl (loading solvent).

4.5.4.3.2 Sample loading and elution

The combined labelled samples were reduced to 50µl and diluted 5 times to 250µl with solvent A. The diluted sample was loaded onto the SAX column with syringe pressure. The flow through was collected and the column washed with 20 column volumes (4ml) solvent A. The flow through from the column was combined with the wash solvent. The sample was step-wise eluted from the column using 1, 2, 5, 10, 20, 37.5, 50, 75, 100, 200,

500mM, 1M and 2M sodium perchlorate (NaClO₄) in solvent A. The fractions were collected in 10ml glass vials. The samples were dried under vacuum.

4.5.4.3.3 Sample clean-up after first dimension chromatography

Solid phase extraction columns were prepared by cutting Pasteur pipettes to accommodate 10mg RP C18 material. The material was equilibrated with 2% ACN/water; 0.1% TEA (acetonitrile, Burdick and Jackson; triethylamine acid; Sigma, loading solvent). The peptide fractions were diluted five times with loading solvent and applied to the columns. After washing with 5 column volumes (100µl) loading solvent, the peptides were eluted stepwise with 5 column volumes 30%, 50% and 70% ACN/water; 0.1% TEA. The eluted fractions were reduced to 50µl, after which 100µl of water was added before the volume was again reduced to 15µl for second dimension separation.

4.5.4.3.4 Second dimension chromatography

Second dimension chromatography was performed on a Dionex Ultimate 3000 nano-HPLC system. The solvent system employed was solvent A, 2% ACN/water; 0.15% TFA and solvent B 80% ACN/water; 0.12% TFA. The gradient was generated at 300nL/min as follows 0-10 minutes 5-15% B, 10-85 minutes 15-45% B, 85 -90 minutes 45-55% B, 90-100minutes 90% B and 100-120minutes 5%B. The gradient from 15-45%B was generated using Chromeleon non-linear gradient 6. Fraction collection commenced after 15 minutes with 100minute duration. The eluted peptides were spotted onto aMALDI target plate using a Probot (LC packings) robotic spotter. MALDI matrix (7.5mg/ml CHCA, 10% 3-hydroxy-4nitro-benzoic acid (at 7.5mg/ml); calibration mixture in solvent B) was added at a 1:2 ratio to the column outflow (600 µl/min) through a micro-T connector. Final matrix concentration was 5mg/ml. Spots were collected at 15second intervals.

4.5.4.3.5 Mass spectrometry

Confirmation of peptide labelling was performed by manually spotting 1 μ l of the sample mixed with CHCA (7.5mg/ml in solvent B) in a 1:1 ratio. After plate model and default calibration update, the samples were ablated at 3800 arbitrary laser intensity units. A total of 500 sub-spectra were used to generate each final spectrum. The grid voltage was 16 kV. Tandem mass spectrometry (MSMS) was performed on the sample with ion selection criteria as 5 precursors per spot, the strongest precursors were first selected and the adducts of 22.99 were excluded.

An exclusion list was created using the mass list from calibration spectra. A total of 2500 shots/spectrum was acquired with an arbitrary laser intensity of 4800. Confirmation of peptides in each fraction was performed by manually spotting 5% of the sample mixed with CHCA (7.5mg/ml in solvent B) in a 1:1 ratio. After plate model and default calibration update, the samples were ablated at 6000 arbitrary laser intensity units. A total of 1000 sub-spectra were used to generate each final spectrum. Mass spectrometry on collected fractions was performed as follows: laser set at 3800 arbitrary units, 500 laser shots/spectrum, and grid voltage 16kV. All spots were internally calibrated using a 5 point calibration mixture with minimum of 1 point to match with mass tolerance of $m/z = 0.1$, maximum outlier error of 10ppm. The interpretation method used for ion selection was: minimum signal/noise ratio = 35, exclude adducts = 22.99, global exclusion list were generated from the calibration spectra with m/z tolerance equal to 0.1. When additional exclusion list were used these were generated from the precursor ion list with m/z tolerance of 0.1 and retention time tolerance of 2 min. In addition all precursors within a 200 FWHM resolution were excluded. Tandem mass spectrometry acquisition parameters were: laser set at 4800 arbitrary units, a maximum of 2500 laser shots/spectrum, grid

voltage at 16 kV. Stop conditions were set to cease acquisition once the following criteria have been met: 8 peaks with signal to noise ratio equal or greater than $S/N = 100$.

Low molecular weight reporter ions from the mass tags were generated by fragmentation in the mass spectrometer and their intensity was measured; the ratios of which determined the relative or absolute amount of protein in each sample. This approach is now being widely used to compare control cases with different disease states in one experiment.

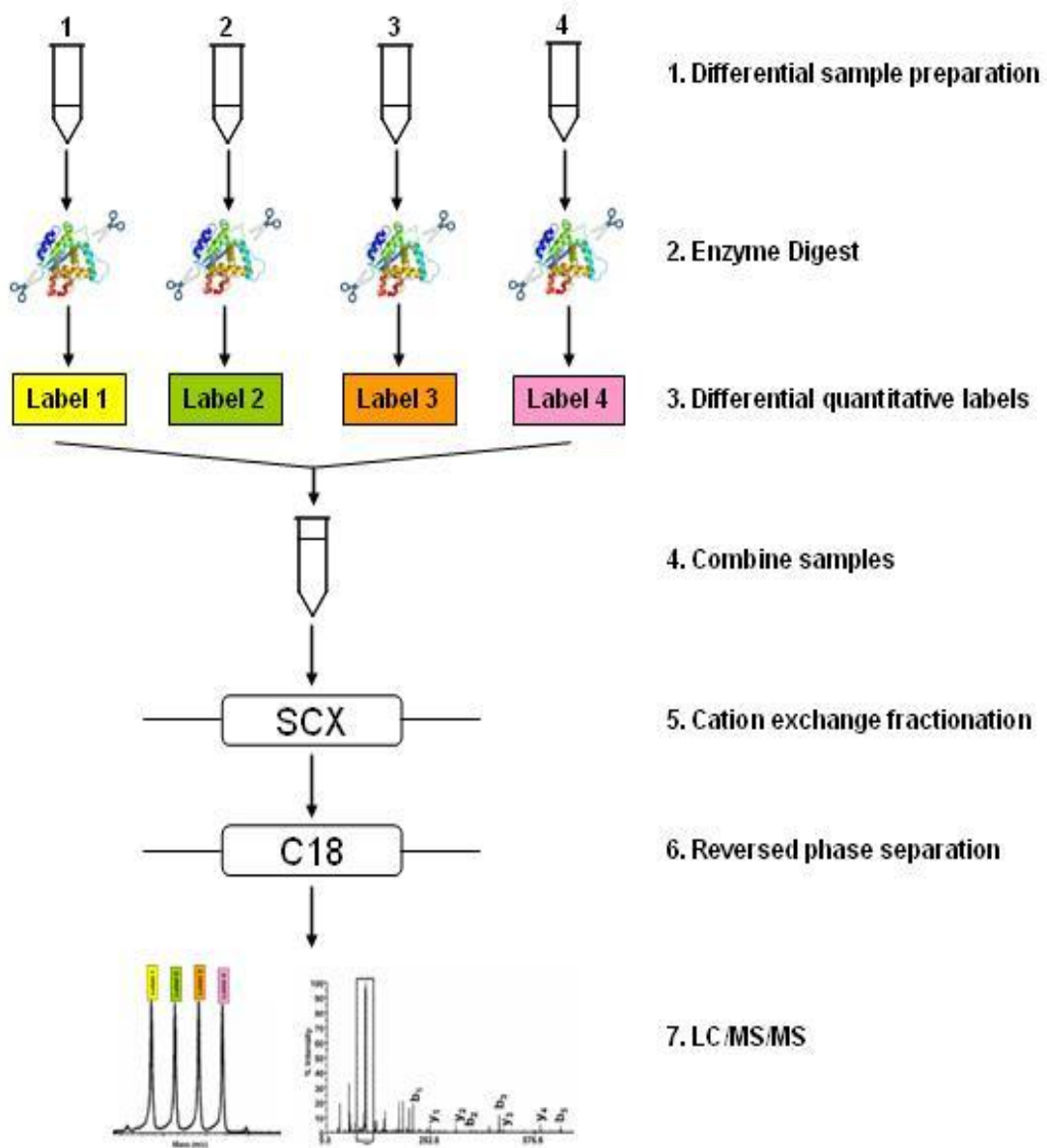


Figure 4.4: Flow chart illustrating the broad concept of iTRAQ and mass

spectrometry. HILIC – hydrophilic interaction chromatography, C18 – a HPLC column that uses C18 substance, LC/MS/MS – liquid chromatography-tandem mass spectrometry

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Chapter 5

Behavioural assessment of rats subjected to fear conditioning (electric foot shock)

5.1 Introduction

Adolescent and adult male Sprague-Dawley rats subjected to footshocks served as our rodent model to study the neurobiology of PTSD. In order to assess the face validity of our model, the present study examined the behaviour of the animals using tools that included the open field, light/dark box test and forced swim test. Once the PTSD-like symptoms were established in the shocked animals, the rats were treated with DCS in subsequent experiments to see if this NMDAR agonist was able to alleviate some of the symptoms.

5.2 Methodology

A group of 90 animals aged 33 days (PND 33) and another group of 90 rats 60 days old (PND 60) were randomly divided into 3 groups of 30 rats each per age group: naïve (N), control+saline (CS) and shock+saline (SS). The aversive stimulus (shock) to which the animals were exposed to consisted of 10 repetitions of footshocks within 1 minute. Contextual fear was measured by observing the freezing behaviour during the time period that animals remained in the shock chamber (see Section 3.1 in Chapter 3 for more detail). To test for associative fear, animals were re-exposed to the shock chamber without further shocks.

Following the assessment of associative fear, animals were placed in a neutral chamber and exposed to a neutral tone for 1 minute. Freezing behaviour was immediately recorded

after the tone has been terminated, and represented non-associative fear. The animals were then subjected to the light/dark box, open field and forced swim tests to evaluate their emotional status.

5.2.1 Statistical analysis

Statistical analyses were done with the assistance of the Centre for Statistical Consultation of the University of Stellenbosch. The software package Statistica was used to perform statistical tests. Shapiro-Wilk normality test indicated that our behavioural data had a Gaussian distribution. Subsequently the behavioural parameters were analyzed using a one or two-way analysis of variance (ANOVA) test that was followed by the Bonferroni *post-hoc* test where appropriate. Where only two groups were analysed, the t-test was used. For experiments where fear extinction was investigated over a 6 day period, repeated ANOVA followed by Bonferroni *post-hoc* test was used. The level of significance was accepted as $p < 0.05$. Data is reported as mean \pm SEM.

5.3 Results

5.3.1 Selection of animals for further investigation and analysis

Freezing time during the associative fear exposure (i.e. second exposure to shock chamber that served as situational reminder) was chosen as the parameter to assess whether animals developed contextual fear. Animals who therefore displayed freezing times greater than that of the mean freezing time of the control group, were selected and included in subsequent analyses. The mean value for our control saline group was 5.12 seconds. All animals that were subjected to footshocks and exhibited freezing times in excess of this value were considered subjects that displayed associative fear.

5.3.2 Emotionality of PND 33 rats: controls (CS) vs shock (SS) (fear conditioned) animals

There were 20 out of a total of 30 animals that spent time exhibiting freezing behaviour greater than controls, when re-exposed to the familiar shock chamber. These animals represented the group of fear conditioned rats. When their freezing behaviour was compared to controls within the neutral chamber/neutral tone scenario, significant differences were observed. The shocked (SS) animals showed significantly higher freezing times when compared to controls (CS) ($20,63 \pm 1,5$ seconds vs $13,38 \pm 2,2$ seconds; $p < 0.02$; t-test), (Figure 5.1)

The emotionality of the shocked animals were then assessed in the light/dark box and compared to controls. Shocked animals spent significantly less time in the light compartment of the light/dark box when compared to controls ($245,77 \pm 8,7$ seconds vs $207,31 \pm 10,5$ seconds, $p < 0.05$; Bonferroni's Multiple Comparison test) (Figure 5.2). This result was inversed when the times spent in the dark compartment of the light/dark box was compared between the two groups ($354,23 \pm 8,7$ vs $392,7 \pm 8,7$ seconds, $p < 0.05$; Bonferroni's Multiple Comparison test) (Figure 5.2).

The prevalence of anxiety-like behaviour and the locomotor activity of the animals were determined in the open field test. There was no significant difference in time spent in the inner or outer zone of the open field test between the shocked (SS) animals and their controls (CS) (Figure 5.3). Similarly there was no significant difference in the total distance travelled in the open field between the two groups (Figure 5.4).

A significant difference was observed in the immobilization time between control rats (CS) and shocked (SS) animals, with the latter having markedly greater immobilization times than the former ($135,4 \pm 10,4$ vs $168,0 \pm 8,6$ seconds; $p < 0.03$, t-test) (Figure 5.5).

5.3.3 Fear extinction in PND 33 control and shocked rats

On the first day of fear extinction testing, animals that were fear conditioned displayed a significantly greater time freezing when they were placed back into the shock chamber in comparison to control animals that were not shocked ($22,7 \pm 2,3$ vs $18,4 \pm 1,3$ seconds, $p < 0.0001$; Repeated measures ANOVA followed by Bonferroni's test) (Figure 5.6). While the time spent freezing decreased by both groups over the next 6 days, the significant difference between the groups was maintained (Figure 5.6).

5.3.4 Emotionality of PND 60 rats: controls (CS) vs shock (SS) (fear conditioned) animals

The mean freezing time of control rats were $42,1 \pm 0,8$ seconds when they were re-exposed to the shock chamber. This amount of freezing time was significantly higher when compared to that of PND 33 rats. There were 16 rats that received foot shock that displayed freezing times greater than this and these animals represented our fear conditioned group for PND 60. Their behaviour was subsequently analysed.

There was no significant difference between the freezing times of shocked animals and controls, when assessed in a neutral chamber with associated neutral tone (Figure 5.7).

There was also no significant difference between the two groups of animals in terms of the time spent in either the light or dark compartment of the light/dark box (Figure 5.8).

When the emotionality and locomotor activity of control and shocked animals were determined in the open field test, no significant differences were observed with respect to time spent in the inner or outer zone (Figure 5.9). However rats that were shocked travelled a significantly greater distance than control rats ($1092,0 \pm 57,3$ vs $824,2 \pm 10,7$ meters, $p < 0.0001$) (Figure 5.10). In the forced swim test, animals that were shocked exhibited immobilization times similar to control rats (Figure 5.11).

5.3.5 Fear extinction in PND 60 control and shocked rats

There was a significant difference in freezing time between control and shocked animals on the first day of fear extinction testing, with shocked animals displaying significantly greater freezing times than controls ($31,6 \pm 2,5$ vs $27,1 \pm 1,0$ seconds, $p < 0.0006$; Repeated measures ANOVA followed by Bonferroni's test) (Figure 5.12). While the time spent freezing decreased by both groups over the next 6 days, the difference between the two groups increased (Figure 5.12). The main reason for the diverging graphs is the slower rate to decrease in freezing time observed in shocked rats on subsequent days of fear extinction testing.

5.4 Discussion

The aim of this section of the overall study was to establish and optimize a rat model of PTSD based on the methodology of Siegmund and Wotjak (2007). PTSD development is associated with learned fear conditioned responses which serve as a reminder of the

traumatic event. PTSD is generally studied via Pavlovian fear conditioning models in many species (Kim & Jung, 2006). Specifically, contextual fear conditioning plays a crucial modulatory role in fear directly associated with stressful events (Lovibond et al., 1984) and thus is considered an associative learning procedure. Additional research has confirmed the relevance of fear conditioning in studying memory processes in PTSD (Kamprath & Wotjak, 2004; Siegmund & Wotjak, 2007).

In 2007, Siegmund and Wotjak developed a mouse model which studied both the associative and non-associative memory processes implicated in PTSD. With the present study we wanted to establish a rat PTSD model and thus we modified the mouse model of Siegmund and Wotjak to fit our species. Our fear conditioning model consisted of applying a foot shock, in a certain context, to elicit a fear response. We increased the intensity and duration of the electric foot shock in order to elicit a valid fear conditioned response in the rats i.e. 10 repetitive foot shocks (each lasting one second) administered over a one minute period (rat model) versus the two second foot shock (mouse model).

Context-specific freezing has been suggested as a main risk factor for development of anxiety disorders (Fanselow, 2000; Buss et al., 2004). In particular, freezing has been identified as an experimental criterion required for PTSD diagnosis in animals (Nemeroff et al., 2006; Siegmund & Wotjak, 2007; Yehuda & LeDoux, 2007). Thus we decided to use freezing time as a dependent parameter of fear to indicate the conditioned fear response to a traumatic stimulus.

PTSD has been described as a fear based disorder and various animal models of fear conditioning exist, such as time-dependent sensitization (TDS) (Liberzon et al., 1997), underwater trauma (Richter-Levin, 1998), predator exposure (Adamec & Shallow, 1993;

Coplan et al., 1996) and early developmental stress (Heim & Nemeroff, 2001; Coplan et al., 1996). We decided to utilize foot shock (Pynoos et al., 1996) as an aversive stimulus in an attempt to model long-term behavioural responses seen in PTSD patients. Previous animal models suggested foot shock as a valuable and relevant fear conditioning protocol (Blanchard & Blanchard 1969; Fanselow 1980; LeDoux et al. 1988; Carrive 2000; Siegmund & Wotjak, 2007).

In the present study, 30 adolescent and 30 adult rats were subjected to the shock protocol. 20 of the adolescent and 16 adult animals were found to exhibit fear conditioning as elicited by an increased freezing response in comparison to control rats that have not been shocked. The behaviour of these selected animals were subsequently analysed in the characterization of our PTSD model. The main finding was that adolescent rats, exposed to shock, displayed clear signs of anxiety-like and depression-like behaviour. This was suggested by a decrease in time spent in the light compartment of the light/dark box, increased immobility times in the forced swim test and significantly higher freezing times during fear extinction. The current data further showed that exposure of adult rats to the same foot shock parameters, generated a milder form of behavioural abnormality with only hyperactivity being recorded in the open field test and poorer fear extinction being observed over a 6 day period.

In general our findings correlate with previous rat studies of contextual fear conditioning, which demonstrated freezing immobility in fear conditioned rats (Fanselow, 1980; LeDoux et al., 1998). Furthermore, our data suggests that our model rendered fear conditioned rats fearful. In line with molecular studies of memory utilizing contextual fear conditioning, we can confirm that context-specific freezing is a major risk factor for anxiety (Grillon, 2002).

Contextual fear conditioning involves placement of the animal in a novel environment (e.g. shock box) and an US (shock) follows. Studies have demonstrated that rats tested in a contextual fear conditioning protocol, learn two associations. First is the association between the training environment (shock box) and the shock (US). The rats learn that the context poses a threat in the form of an unsignalled shock. Once this association is formed, subsequent visits to the shock box itself, even in the absence of a shock, invoke a conditioned fear response in the form of freezing. Therefore to test for memory retention, our animals were re-exposed to the shock chamber in the absence of the foot shock and their freezing time was recorded. We found that the majority of rats of both age groups, when re-exposed to the context in the absence of the foot shock, displayed freezing times greater than their respective controls.

Associative memory relates to the memory of the trauma (i.e. avoidance and re-experiencing). In our model, the fear memory was behaviourally expressed as an increased freezing response (Blanchard & Blanchard, 1969) in fear conditioned rats. This finding demonstrated an environment-shock association by the fear conditioned rats. During the fear conditioning rats learned that the context predicts the aversive foot shock. We suggest that upon re-exposure to the context, the fear-related memory was triggered and manifested as an increased freezing response in the fear conditioned animals. Therefore it can be concluded that the duration spent in the context environment was adequate to shape and store the contextual representation as fear conditioned rats retained the fear memory associated with the context.

Clinically, PTSD patients exhibit behavioural sensitization to stress (Servatius et al., 1995) and an over-generalized fear to situations normally considered safe (Wesa & Flor, 2007). Over-generalized fear is recognized as one of the cardinal symptoms of PTSD and

represents itself as an autonomic hyperarousal state. Hyperarousal correlates to those memories which lack association to the trauma (i.e. non-associative memories). To test for a non-associative fear memory, we subsequently exposed rats to a tone in a novel context (Siegmund & Wotjak, 2007) and once more recorded the rats' freezing response. The present study observed that adolescent rats exhibited significantly higher levels of freezing than their controls. This difference was less apparent in the adult group of animals. The data nevertheless suggested that the fear conditioned rats were more sensitized to the tone. This finding correlates to clinical PTSD studies demonstrating behavioural sensitization to stress (Servatius et al., 1995). The shocked rats thus displayed hypersensitivity which is in accordance with clinical observations of hyperarousal in PTSD patients (APA, 1994; 2000; 2013). Additionally, when comparing the associative to the non-associative fear memory, fear conditioned rats exhibited a greater fear to the tone. This result indicates that our fear conditioning model elicits more than one PTSD-like symptom, most evidently in the adolescent group.

Dysregulated hippocampal-dependent mechanisms have been shown to contribute to the putative fear learning abnormalities and sensitization reported in PTSD (Tsetsenis et al., 2007). In addition, animal fear conditioning studies have implicated the HC in mediating conditioned fear responses by inducing LTP. Hippocampal LTP is a form of synaptic plasticity known to be involved in declarative memory (memory of facts and events). Behavioural results of the current fear conditioning model confirmed the role of learning and memory mechanisms involved in the development and maintenance of fear acquisition. Results demonstrated that during fear conditioning the rats learned to associate the context with the aversive stimulus. Whereas the associative test (re-exposure to shock chamber) indicated that fear conditioned rats recalled the traumatic event i.e. a function of memory retrieval.

A brief explanation on how the sensory information of the foot shock is transmitted by synapses along the neural pathway and stored in short-term memory follows. During the association/learning process the excitatory neurotransmitter, glutamate is released which binds to and opens the hippocampal NMDARs. Opening of the channels allows an influx of Ca^{2+} which triggers LTP. A more detailed discussion on the neurochemistry is provided in Chapter 6.

Fear conditioning leads to PTSD- and depression-like behaviour

To further test for various signs of emotional distress, the following behavioural tests were employed, namely the light/dark avoidance test, open field test and the forced swim test. Anxiety-like responses and locomotor activity are commonly measured with the light/dark avoidance test and the open field test (Crawley & Goodwin, 1980; Prut & Belzung, 2003; Wolf & Frye, 2007; Adamec et al., 2010; Cacciaglia et al., 2013). These two tests create conflicting innate tendencies of avoidance and exploring the arenas, as rodents have a natural desire to explore a novel environment yet at the same time seek shelter/avoid the unfamiliar. Depression-like behaviours in rats are commonly measured with the forced swim test which provides insights into the level of helplessness in rodents (Ji et al., 2014). The rationale for employing the forced swim test was based on people exhibiting helplessness after experiencing a traumatic event (Asmundson & Taylor, 2009).

The light compartment of the light/dark avoidance test serves as an aversive novel space (Crawley & Goodwin, 1980). In our study, we found that fear conditioned adolescent rats showed preference for remaining in the dark compartment of the light/dark avoidance test, as indicated by their increased time spent there when compared to control rats. This data suggested that these fear conditioned animals experienced the light compartment as

potentially dangerous which led them to choose the more protected area of the dark compartment. As previously mentioned, the light/dark avoidance test indicates heightened anxiety-like behaviour (Cacciaglia et al., 2013; Adamec et al., 2010). It was therefore concluded that our fear conditioned adolescent rats were anxious, a result that was in line with prior reports of PTSD subjects exhibiting abnormally high conditioned fear responses (Orr et al., 1993).

On the other hand open field data showed that fear conditioned adult rats travelled greater distances than their control counterparts. This observation suggested that the fear conditioned animals were hyperactive and reflecting an increased state of hyperarousal and anxiety in the rats (Miao et al., 2014; Qiu et al., 2013). The stressor in the forced swimtest is inescapable and affected rats often exhibit signs of helplessness during this test. It is commonly accepted that measurements of immobility represented depressive-like behaviour (Shaldubina et al., 2005). Generally, the forced swim test is a two session test administered twenty-four hours apart (Shaldubina et al., 2005). We decided to use a one session test as previous studies demonstrated that stressors produce depression-like states amongst groups during the first session (Zhong et al., 2014). Fear conditioning resulted in increased immobility which suggests that these rats elicited a depressive-like behaviour in these rats. The present model thus elicited depressive-like symptoms in fear conditioned rats. The forced swim test behaviour was interpreted as evidence complementary and supportive of our anxiety-like behaviours as PTSD and depression frequently co-occur among psychiatric patients.

In summary, animals subjected to fear conditioning displayed behavioural changes indicative of anxiety and depression, thereby granting good face validity to our model. The

behavioural findings of the current study are consistent with other animal models of fear conditioning as well as clinical findings in human PTSD. We were therefore comfortable to use our model in subsequent investigations.

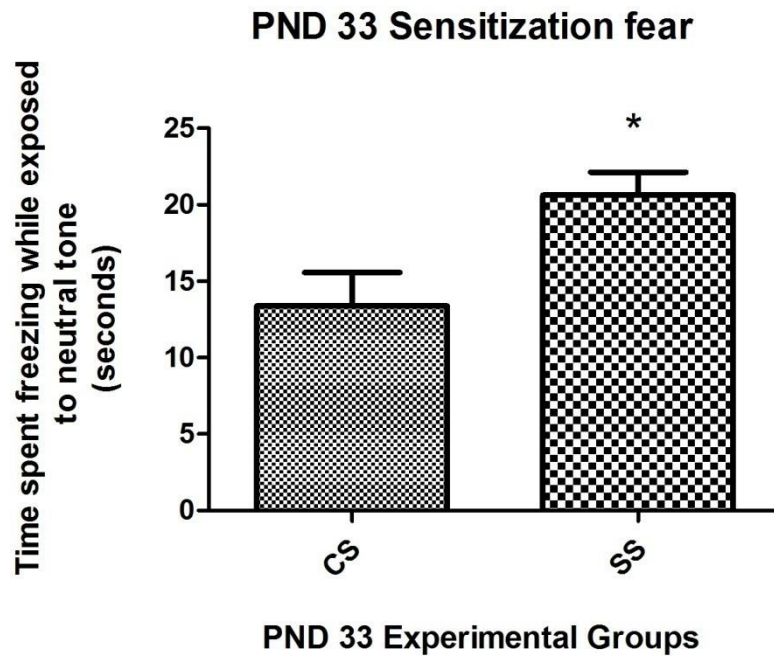


Figure 5.1: Histograms depicting time spent freezing while exposed to neutral tone in control and shocked animals of PND 33. PND – post natal day, CS – control+saline, SS – shock+saline. Values are expressed as mean±SEM (n=20/group) in time (seconds).

*p≤0.05

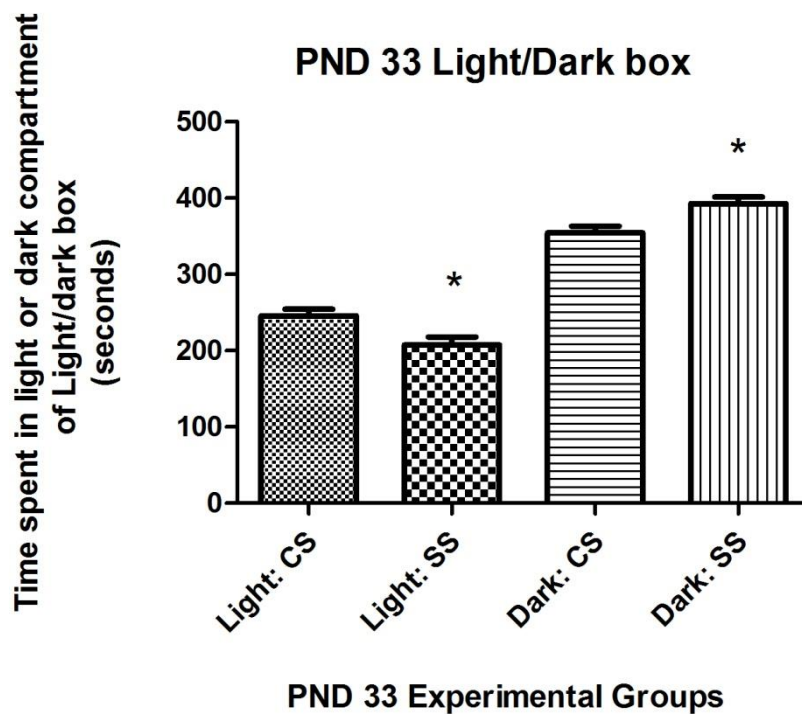


Figure 5.2: Histograms depicting the time spent in the light/dark box in control and shocked animals of PND 33. PND – post natal day, CS – control+saline, SS – shock+saline. Values are expressed as mean±SEM (n=20/group) in time (seconds).

*p≤0.05

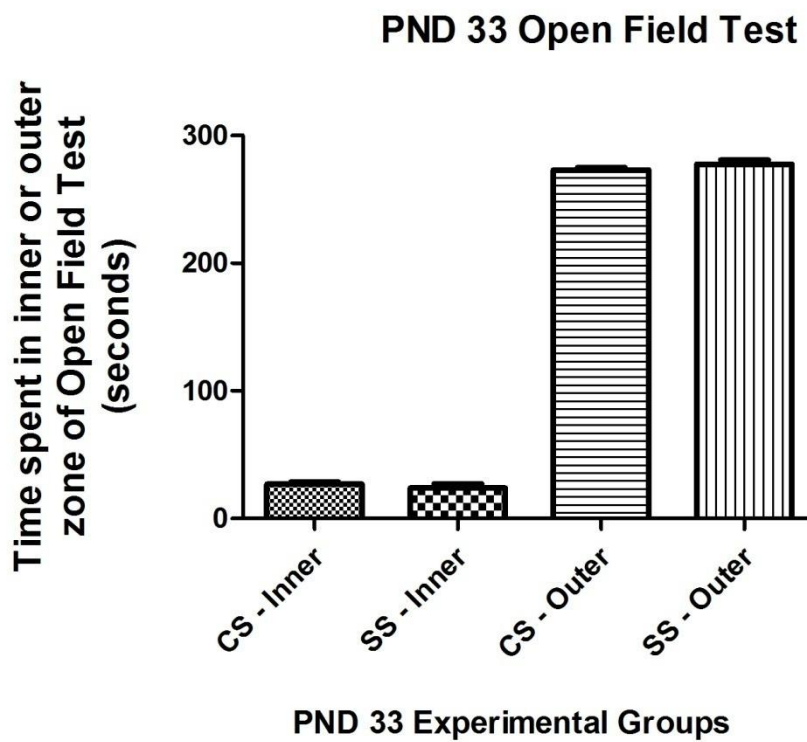


Figure 5.3: Histograms depicting the time spent in the open field test in control and shocked animals of PND 33. PND – post natal day, CS – control+saline, SS – shock+saline. Values are expressed as mean±SEM (n=20/group) in time (seconds).

*p≤0.05

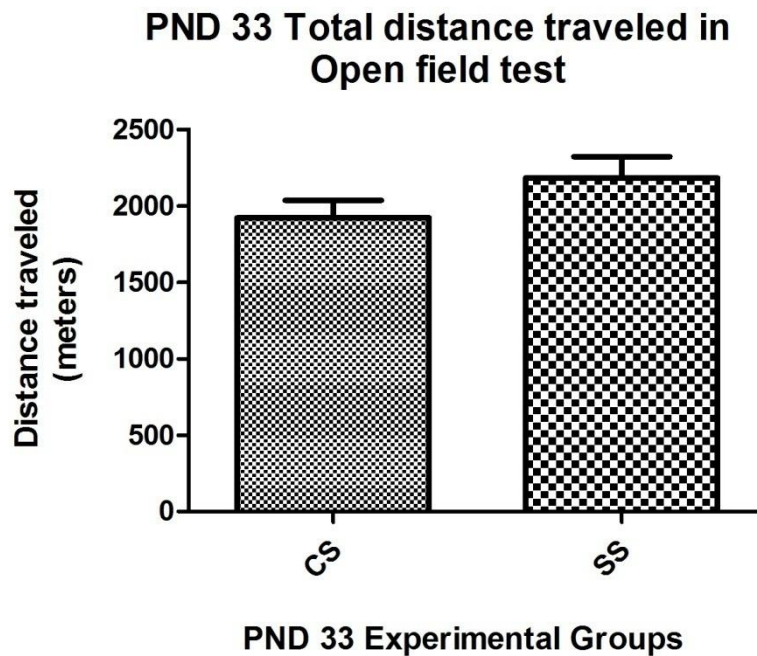


Figure 5.4: Histograms depicting the total distance travelled in the open field in control and shocked animals of PND 33. PND – post natal day, CS – control+saline, SS – shock+saline. Values are expressed as mean \pm SEM (n=20/group) in time (meters). *p \leq 0.05

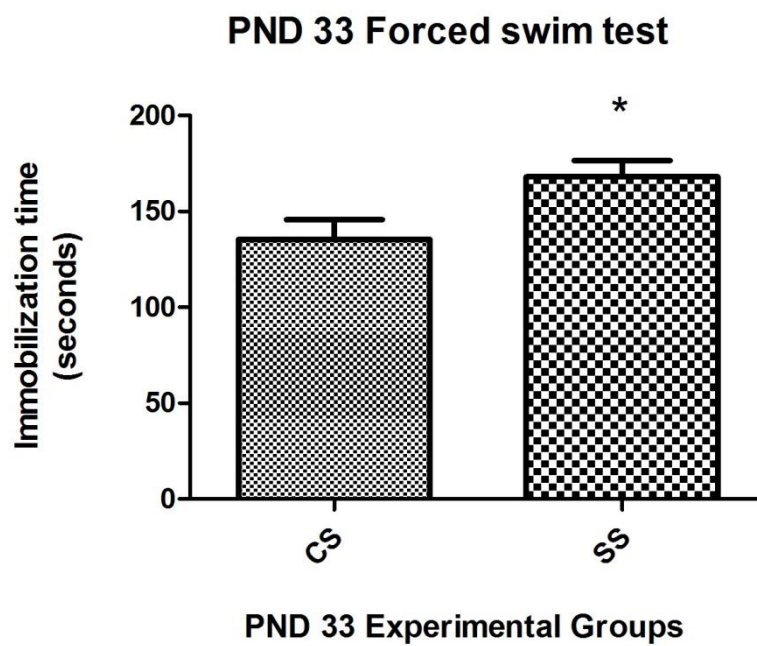


Figure 5.5: Histograms depicting the time spent immobilized in the forced swim test in control and shocked animals of PND 33. PND – post natal day, CS – control+saline, SS – shock+saline. Values are expressed as mean±SEM (n=20/group) in time (seconds).

*p≤0.05

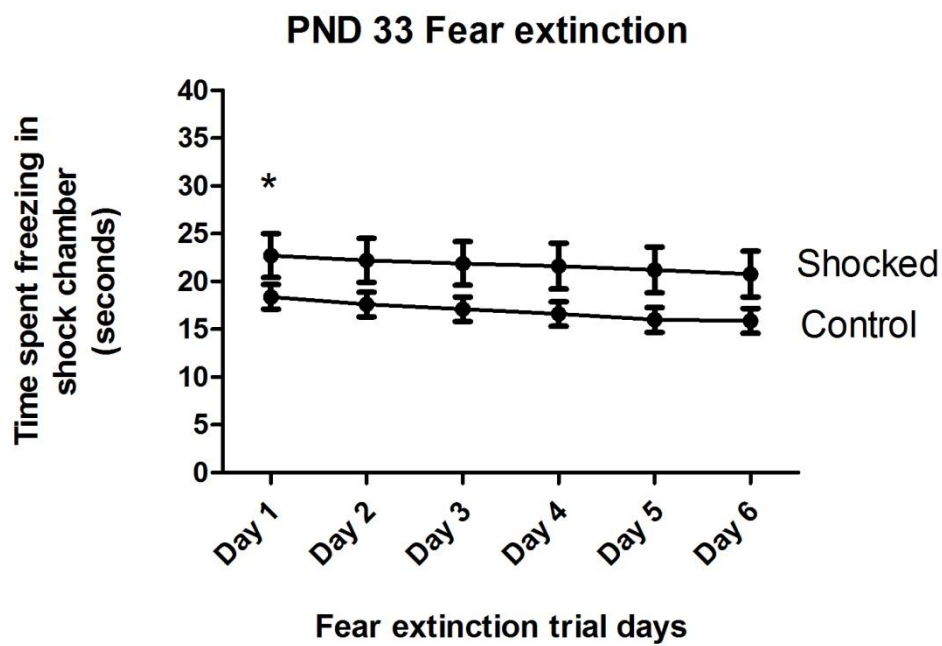


Figure 5.6: A line graph depicting the time spent freezing over the course of the fear extinction trial days in control and shocked animals of PND 33. PND – post natal day. Values are expressed as mean \pm SEM (n=20/group) in time (seconds). *p<0.0001

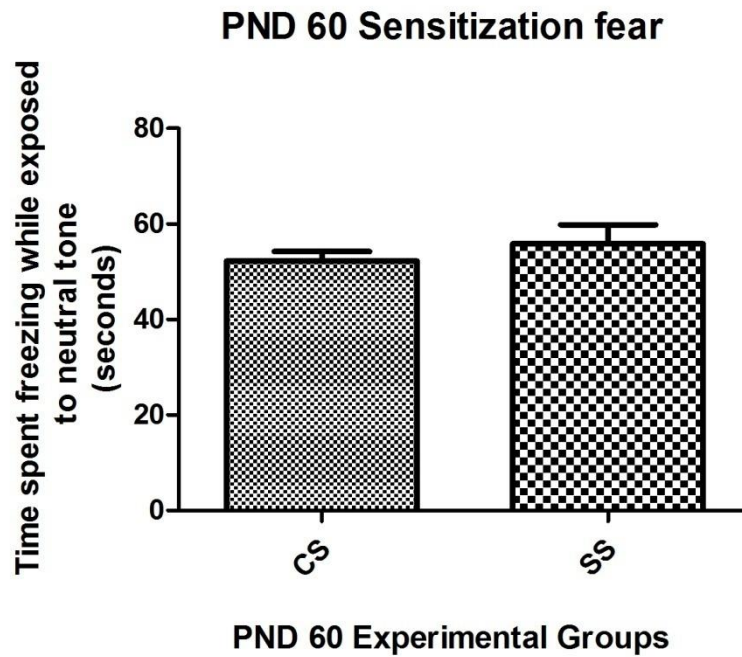


Figure 5.7: Histograms depicting time spent freezing while exposed to neutral tone in control and shocked animals of PND 60. PND – post natal day, CS – control+saline, SS – shock+saline. Values are expressed as mean \pm SEM (n=16/group) in time (seconds).

*p \leq 0.05

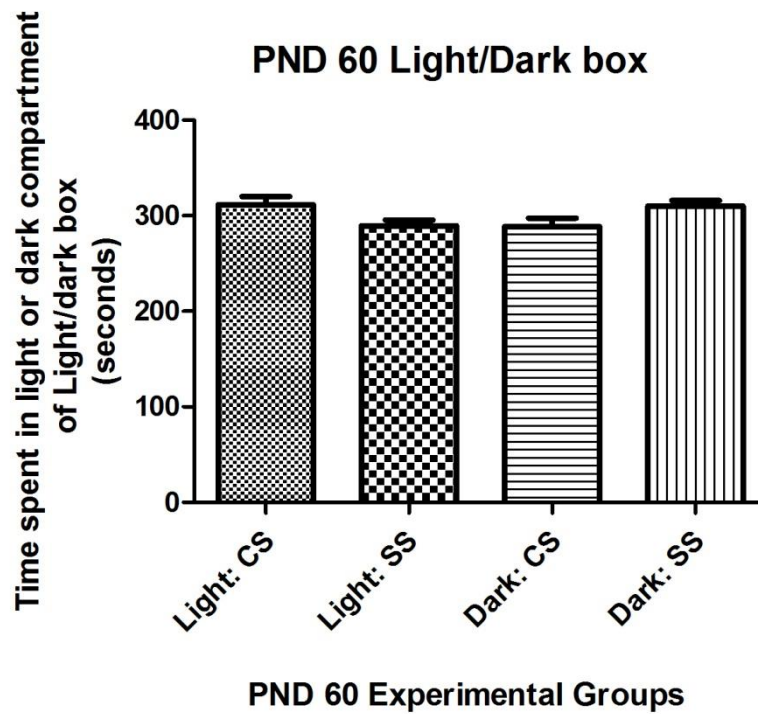


Figure 5.8: Histograms depicting time spent in the light/dark box in control and shocked animals of PND 60. PND – post natal day, CS – control+saline, SS – shock+saline. Values are expressed as mean±SEM (n=16/group) in time (seconds). *p≤0.05

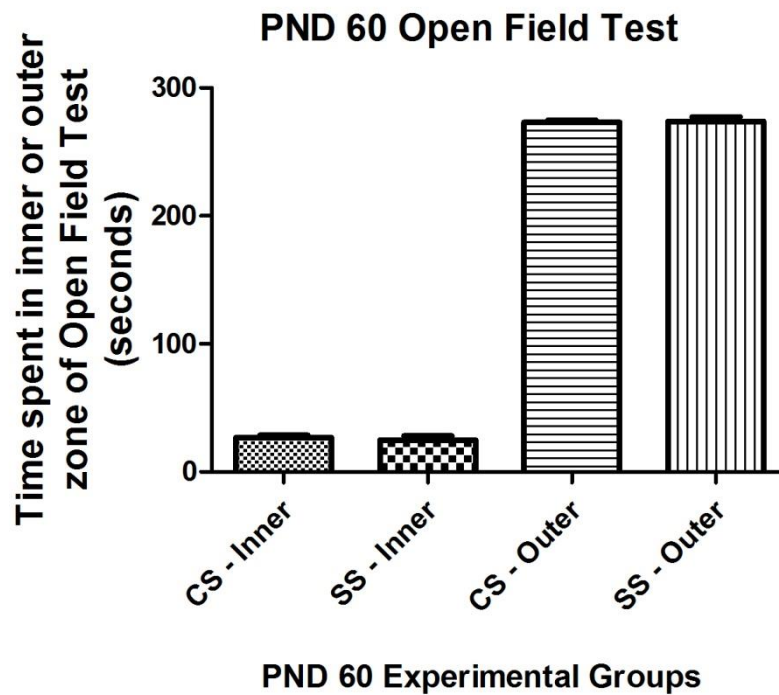


Figure 5.9: Histograms depicting time spent in the open field test in control and shocked animals of PND 60. PND – post natal day, CS – control+saline, SS – shock+saline. Values are expressed as mean±SEM (n=16/group) in time (seconds). *p≤0.05

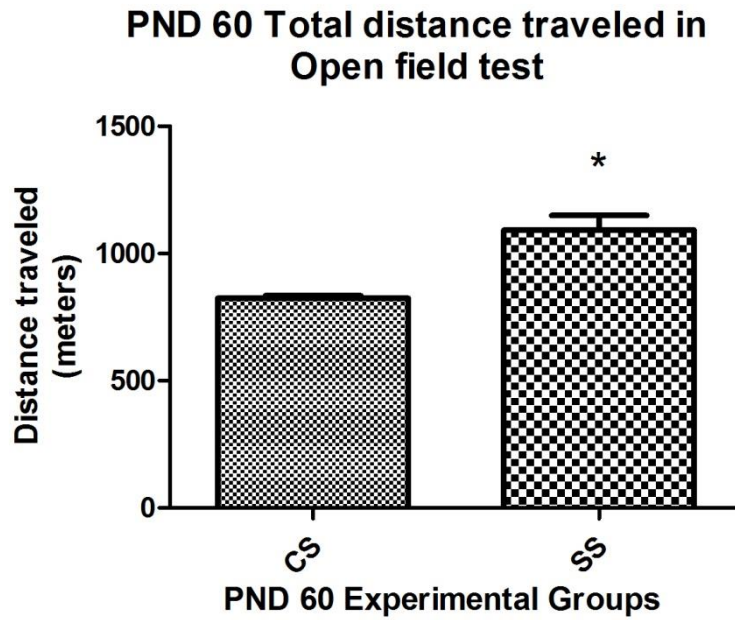


Figure 5.10: Histograms depicting time total distance travelled in control and shocked animals of PND 60. PND – post natal day, CS – control+saline, SS – shock+saline. Values are expressed as mean±SEM (n=16/group) in time (meters). *p≤0.0001

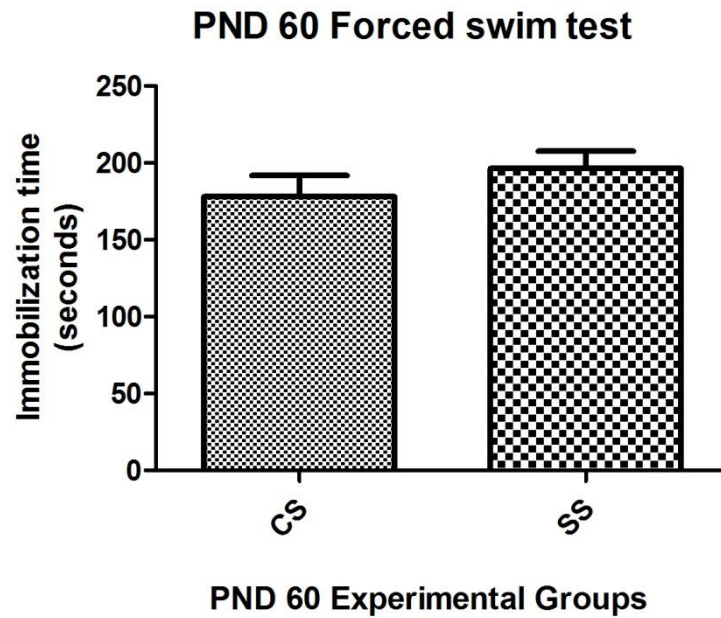


Figure 5.11: Histograms depicting time spent immobilized in the forced swim test in control and shocked animals of PND 60. PND – post natal day, CS – control+saline, SS – shock+saline. Values are expressed as mean \pm SEM (n=16/group) in time (seconds).

*p \leq 0.05

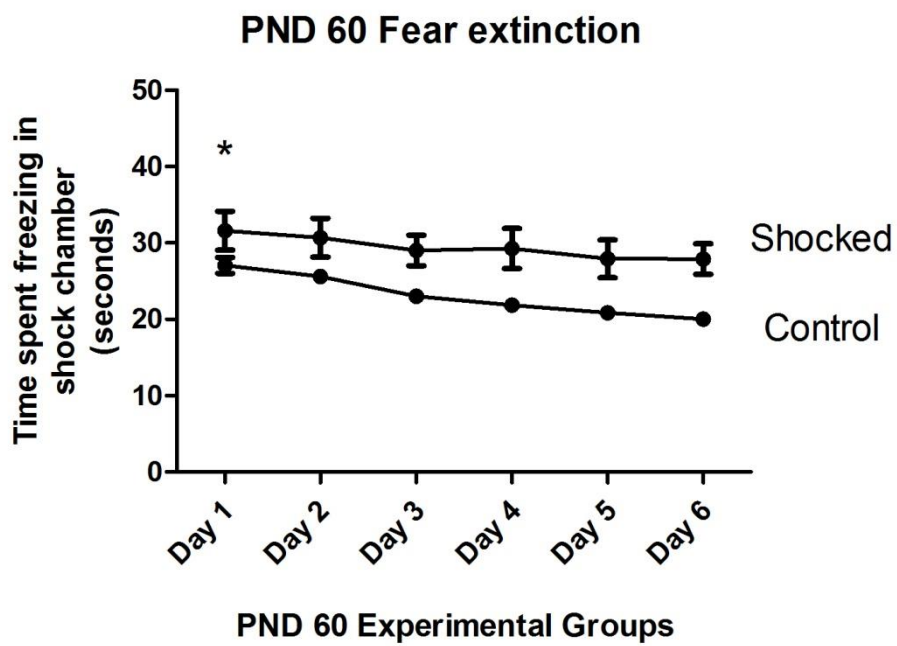


Figure 5.12: A line graph depicting the time spent freezing over the course of the fear extinction trial days in control and shocked animals of PND 60. PND – post natal day. Values are expressed as mean \pm SEM (n=16/group) in time (seconds). *p \leq 0.0006

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Chapter 6

An investigation into whether intrahippocampal DCS infusion can reverse the PTSD phenotype as displayed by our rat PTSD model

6.1 Introduction

We exposed adolescent and adult rats to electric footshock, and observed that these animals displayed behaviours akin to PTSD-like symptoms. The HC is known to play a major role in memory as well as in emotional processing. With their “spatial map” theory, O’Keefe and Nadel (1978) posited a general role for the HC in the spatial processing of environmental stimuli. This theory prompted researchers to focus specifically on the involvement of the HC in fear acquisition, which led them to delineate a role for the HC in contextual fear conditioning (Phillips & LeDoux, 1992; Anagnostaras et al., 1995; Winocur, 1997; Frankland et al., 1998; McDonald et al., 2002) and fear extinction (Szapiro et al., 2003; Barad, 2005). Subsequent experiments therefore concentrated on the HC as a pivotal brain area in establishing anxiety-like behaviours. Since evidence has been provided for the role of hippocampal glutamate receptors in the pathology and treatment of anxiety (Krystal et al., 2002; Stewart & Reid, 2002), manipulation of these receptors seemed an appropriate strategy to adopt to investigate ways of reversing the PTSD-like symptoms in our animal model. As such we examined the potential of the NMDAR agonist DCS, to serve as an anti-PTSD therapy.

D-cycloserine is an antibiotic and partial NMDAR1 agonist at the glycine site on the NMDAR1 subunit and has been found to be effective in facilitating extinction learning in rats when administered before or immediately after extinction training (Ledgerwood et al.,

2003; 2005; Walker et al., 2002; Yang & Lu, 2005; Philbert et al., 2013). While these studies suggest DCS to have beneficial effects for PTSD, its exact mechanisms of action post NMDAR binding remains to be elucidated. The purpose of this section of the study was therefore to establish whether intrahippocampal DCS infusion can reverse the PTSD phenotype as displayed by our rat PTSD model prior to investigating its molecular mechanism of action which is described in subsequent chapters.

6.2 Methodology

A total of 240 (120 adolescent and 120 adult) rats were used in this experiment. Animals were subjected to a single bout of electric foot shock as previously described (Section 3 of Chapter 4 for more detail) and their freezing behaviour was documented. Some animals (control+DCS and fear conditioned+DCS) had indwelling cannulae inserted into their dorsal hippocampi to facilitate the infusion of either saline or DCS (15 mg/kg) for 6 consecutive days (see Section 4 in Chapter 4 for more detail). The emotional states of all the animals were then assessed 24 hours after the last saline or DCS infusion.

6.2.1 Statistical analysis

Similar to Chapter 5, here too the statistical analyses were done with the assistance of the Centre for Statistical Consultation of the University of Stellenbosch. The software package Statistica was used to perform statistical tests. Shapiro-Wilk normality test indicated that our behavioural data had a Gaussian distribution. Subsequently the behavioural parameters were analyzed using a one or two-way ANOVA test that was followed by the Bonferroni's Multiple comparison test where appropriate. Where only two groups were analysed, the t-test was used. For experiments where fear extinction was investigated over

a 6 day period, repeated ANOVA followed by Bonferroni's Multiple Comparison test was used. The level of significance was accepted as $p < 0.05$. Data is reported as mean \pm SEM.

6.3 Results

6.3.1 Associative fear memory induced by fear conditioning context

No significant difference was noted in the amount of freezing after exposure to the aversive stimulus at each of the age groups studied (Table 6.1). There was a tendency for fear conditioned (shock+saline and shock+DCS) animals to freeze more than their respective controls (control+saline and control+DCS), but this was not significant. There was however a significant difference in freezing time between PND 33 and PND 60 rats in all the respective groups (Table 6.1; $p < 0.05$, Bonferroni's Multiple Comparison test).

There was no significant increase in freezing behaviour between fear conditioned (SS) and control PND 33 rats (CS) when re-exposed to the context (Table 6.2). The freezing time of control animals treated with DCS (CD) also did not differ significantly from that of control saline treated (CS) animals. On the other hand fear conditioned animals that were treated with DCS (SD) had significantly lower freezing times than their respective controls (CD) (Table 6.2; $p < 0.05$, Bonferroni's Multiple comparison test).

Overall the freezing times of adult rats were significantly higher than adolescent rats (Table 6.2). Similar to adolescent rats, the freezing times of shocked adult rats (SS) did not differ significantly from their respective controls (CS) (Table 6.2). However, shocked rats

that were given DCS (SD) displayed significantly shorter freezing times than their shocked rats given saline (SS) (Table 6.2; $p < 0.05$, Bonferroni's Multiple comparison test).

6.3.2 Fear conditioned rats displayed a greater sensitized fear response

Adolescent rats exposed to fear conditioning (SS) exhibited a significantly higher freezing time than their control (CS) when exposed to a tone in a neutral chamber (Figure 6.1; Table 6.3; $p < 0.05$, Bonferroni's Multiple comparison test). However this increase in freezing time was not seen in shocked rats that received DCS treatment (SD), with these animals displaying freezing times similar to controls (Figure 6.1; Table 6.3).

While the adult group of shocked rats also had higher freezing times than their respective controls, this increase was not significant (Figure 6.1; Table 6.3). However all animals that were treated with DCS (CD and SD) exhibited significantly shorter freezing times than their respective controls (CS and SS) (Figure 6.1; Table 6.3; $p < 0.05$, Bonferroni's Multiple comparison test).

6.3.3 Anxiety-like symptoms measured in the light/dark box

In the adolescent group naïve animals, saline-treated control animals (CS) and shocked animals (SS) spent significantly shorter time in the light compartment than the rats that received DCS (CD and SD) (Figure 6.2; $p < 0.05$, Bonferroni's Multiple comparison test). A similar pattern of time spent in the light compartment was observed in the adult group of animals, but the difference between controls and DCS treated animals were less pronounced (Figure 6.2). The time spent in the dark compartment of the light/dark box

complemented these results. The naïve and saline groups spent more time in the dark compartment when compared to the DCS groups (Figure 6.3), with the fear conditioned group (shock + saline) spending the greatest amount of time compared to all other groups, in the dark, secure compartment ($p < 0.05$, Figure 6.3).

6.3.4 Anxiety-like symptoms confirmed by the open field test

Post-hoc analysis of the open field complemented the light/dark box results. Bonferroni tests revealed that DCS groups spent less time in the outer zone when compared to the saline and naïve groups, with shocked animals that received DCS treatment doing so significantly (Figure 6.4; $p < 0.05$). DCS groups spent significantly more time in the inner zone when compared to the saline and naïve groups (Figure 6.5; $p < 0.05$). These results were the same for both age groups of animals. A general locomotor effect of the drug treatment, DCS, can be ruled out as there was a discrepancy in the locomotor activity between adolescent and adult rats when compared to their shocked counterparts (Figure 6.7). Adolescent DCS-treated rats traveled significantly greater distances than the shocked adolescents, whereas adult DCS-treated rats traveled significantly less than the shocked adults (Figure 6.7; $p < 0.05$).

The freezing responses of the adolescent animals in the open field showed that the fear conditioned group (SS) froze significantly more compared to their controls (CS) (Figure 6.6; $p < 0.05$, Bonferroni's Multiple comparison test). Shocked animals that were given DCS (SD) displayed significantly lower freezing when compared to shocked animals that were treated with saline (SS) (Figure 6.6; $p < 0.05$, Bonferroni's Multiple comparison test). The results of the adolescent rats were mirrored in the adult group of animals, but the freezing responses in these animals were to a lesser degree (Figure 6.6).

6.3.5 Anxiety and depressive-like behaviours as measured in forced swim test

The fear conditioned (SS) group of animals was significantly more immobile when compared to the controls (Table 6.4; $p < 0.05$, Bonferroni's Multiple comparison test). Animals that received DCS treatment spent significantly less time being immobile than their respective saline-treated controls (Table 6.4; $p < 0.05$, Bonferroni's Multiple comparison test). This result was evident in both age groups, but more distinct in the adolescent rats.

The fear conditioned (SS) group made more attempts to climb out of the cylinder when compared to all the other groups for both ages. A decrease in climbing attempts was noted in DCS-treated rats, with the fear conditioned+DCS (SD) group displaying the least climbing attempts for both age groups (Table 6.5; $p < 0.05$, Bonferroni's Multiple comparison test).

6.3.6 Tabled summary of behavioural results

Behavioural parameters	Fear conditioning (footshock)	DCS treatment
Associative fear	↑	↓
Non-associative fear	↑	↓
Freezing during extinction trials	↑	↓

6.4 Discussion

6.4.1 *Amelioration of PTSD-like symptoms by DCS treatment*

The NMDA agonist DCS has been shown to be effective as an augmentation agent in exposure-based CBT in humans with GAD, SAD and panic disorder, phobias (Ressler et al., 2004; Hofmann et al., 2006; Kushner et al., 2007; Guastella et al., 2008; Wilhelm et al., 2008; Storch et al., 2010, Otto et al., 2010). In addition, DCS has also been found to reduce the rate of relapse following exposure-based CBT (Richardson et al., 2004).

Separate studies have demonstrated that contextual fear conditioning relies heavily on the HC and that this brain region plays an important role in fear extinction (Szapiro et al., 2003; Barad, 2005). It therefore made logical sense to assess the efficacy of intrahippocampal DCS infusion on fear-related behaviours. The rationale for administering DCS before the onset of the extinction period came from previous studies that showed behavioural differences from drug administration given prior to fear extinction (Walker et al., 2002; Ledgerwood et al., 2003; Yang & Lu, 2005, 2007; Woods & Bouton, 2006; Lee et al., 2006; Mao et al., 2006; 2008; Bouton et al., 2008; Langton & Richardson, 2008; Lin et al., 2008). For the drug dose we consulted previous behavioural studies utilizing DCS in conjunction with fear extinction (Walker et al., 2002; Ledgerwood et al., 2003). This specific DCS dosage of 15mg/kg has been shown to facilitate performance on learning tasks in rodents, a property attributed to NMDAR potentiation (Monahan et al., 1989; Baran et al., 1995; Depoortere et al., 1999; Andersen et al., 2002). Based on previous animal studies, our dosage can be predicted to exert agonistic effects on the glycine site of the NMDAR (Walker et al., 2002; Ledgerwood et al., Yang & Lu, 2005; Woods & Bouton, 2006; Lee et al., 2006; Mao et al., 2006; Yang & Lu, 2007; Mao et al., 2008; Bouton et al., 2008; Langton & Richardson, 2008; Lin et al., 2008).

In the current study a significant effect of treatment was observed in fear conditioned animals. Rats receiving pre-extinction intrahippocampal infusions of DCS displayed lower levels of freezing relative to their controls in both the context and tone exposure. Thus pre-extinction treatment with DCS had an opposing effect to fear conditioning. In addition, a decrease in the time spent freezing in the open field was also noted in DCS-treated rats when compared to the fear conditioned animals. In non-conditioned animals (controls), no significant treatment effect was observed.

Our findings suggest that DCS facilitates extinction of learned fear. The DCS data therefore replicates the initial findings of Walker et al. (2002). The current findings also correlate with the work of Yamamoto et al. (2008), who demonstrated that extinction training in unison with DCS-treatment improved fear extinction in a fear conditioning model of single prolonged stress (SPS). Myers et al. (2011) also showed enhancement of fear extinction when DCS was administered systemically or infused into the rat HC.

The present study confirms that DCS treatment elicits a generalized extinction of fear, as observed in our light/dark box and open field behavioural tests. DCS groups spent significantly more time in the light compartment when compared to the naïve, control and fear conditioned groups, suggesting that the DCS groups were less averse to the novel illuminated area. This increased exploration of the light compartment is suggested as an index of anxiolytic activity. Complementary, the naïve and saline groups spent more time in the dark compartment when compared to the DCS groups, with the fear conditioned group spending significantly more time compared to all other groups in the dark, secure compartment. The results of this test demonstrated the extent to which DCS facilitates exploratory activity/neophobia and was in line with previous reports showing how DCS

aided the treatment of anxiety disorders in the clinical setting (Goldstein et al., 2001; Wrubel et al., 2007; Bredy et al., 2007; Bredy & Barad, 2008; Quirk & Mueller, 2008; Chang et al., 2009; Graham and Richardson, 2009 b; Milad et al., 2009a).

Behavioural analysis in the open field showed fear conditioned rats to exhibit high levels of freezing behaviour. In contrast, animals treated with DCS displayed significantly lower freezing times. This freezing result complemented the data for time spent in the open field. Fear conditioned rats were observed to spend the most time in the outer zone compared to their counterparts; confirming that the fear conditioning paradigm elicits anxious behaviour. Conversely, DCS groups spent significantly more time in the inner zone when compared to the saline-treated and naïve groups, confirming that pre-extinction DCS infusions leads to a reduction in anxiety.

PTSD and depression frequently co-occur among psychiatric patients, leading to increased morbidity and mortality. Considering this co-morbid relationship, it was decided to test whether DCS might have different effects in fear conditioned versus control rats on coping behaviour in a stressful environment, by employing the forced swim test. This test is used to screen preclinical drugs in order to examine their antidepressant properties (Browne & Lucki). The stressor in this test is inescapable and thus rats acquire helplessness during the training session, which contrasts to the open field and light/dark box, where the aversive inner zone and the illuminated compartment is avoidable. Behavioural analysis of the forced swim test data, revealed significant group differences with increased immobility noted in the fear conditioned group compared to controls, whereas rats receiving DCS prior to the test displayed decreased immobility compared to rats given saline. The fear conditioned rats therefore displayed less escape activity and

signs of depression-like behaviour and these behaviours were less evident in DCS treated rats. This finding was consistent with that of Lopes et al. (2007) where the effects of DCS were also examined in the forced swim test.

Interestingly chronic DCS exposure prior to fear conditioning has been shown to decrease the effectiveness of DCS on extinction (Parnas et al., 2005; Lee et al., 2006). The reason for this stems from an apparent temporary change in the function of the glycine/NMDAR complex. This finding supports the hypothesis that fear extinction is a learnt behaviour and therefore interference with the optimal function of NMDARs may impair this behaviour. Earlier experiments by Quartermain et al., (1994) reported that 15 days of continuous DCS treatment resulted in significantly poorer spatial memory retention in mice suggesting that chronic NMDA activation may cause receptor desensitization, thereby impacting negatively on the animal's ability to learn. These findings highlight the importance of the timing as well as the frequency or period of DCS administration when treatment for PTSD is considered. Nevertheless a study performed by Yamamoto et al. (2007), clearly demonstrated that administration of DCS enhanced fear extinction. These seemingly contrasting observations can be attributed to the variations in the experimental procedures of these studies as differences in the amount and duration of DCS exposures have been noted.

The data thus far presented showed that, similar to humans (Breslau et al., 1998), rats display a wide range of responses to trauma and that some animals are much more susceptible than others. This individual variability to the traumatic stimuli added to the validity of our animal model as this finding met the criteria for analogous behaviour. In addition, as was expected, a decrease in the fear response was noted after DCS administration, which reinforced the predictive validity of our fear conditioning paradigm.

Considering the above findings, our fear conditioning paradigm can be regarded as a valid animal model of PTSD as it met the criteria of both face and predictive validity, and construct validity to some degree.

6.4.2 Age differences in the development of PTSD-like symptoms

There is a growing body of literature on the effects of DCS on contextual fear conditioning and extinction learning in rodents (Silverstri & Root, 2008; Yamamoto et al., 2008; Kalisch et al., 2009; Langton et al., 2009; Waddell et al., 2010; Toth et al., 2012; Gupta et al., 2013; Ren et al., 2013). Of these animal studies, only a handful has investigated the adverse effects of trauma in adolescents.

A recent study by McCallum and colleagues (2010) found that systemic DCS injections (dose of 1.0 mg/kg), given post extinction, impaired extinction retention in this population during an extinction test given 24 hours later. By increasing the DCS dosage to 15mg/kg, extinction retention was shown to improve and the adolescent rats completely recovered from their previously exhibited fear. The same authors also demonstrated that by doubling the extinction training and systemic DCS administration, alleviation of impaired extinction was possible (McCallum et al., 2010). In another study a mouse model was used to investigate the effects of hippocampal-dependent contextual fear conditioning and amygdala-dependent cued fear conditioning on fear learning and memory processes in the period into and out of adolescence (Pattwell et al., 2011). Reports that contextual fear learning is present at a very early age i.e. in rats younger than PND 29 (Rudy, 1993; Moriceau & Sullivan, 2006), prompted Pattwell and colleagues (2011) to investigate the absence or suppression of contextual fear in the early-adolescent period. Three different cohorts pertaining to adolescence were used, namely postnatal day (PND) 29, 39, and 49

which correlate to early, mid, and late adolescence, respectively (Spear, 2000; Hefner & Holmes, 2007; Adriani et al., 2004). An adult cohort, PND 70, was used for baseline comparisons. Furthermore they decided to separate their adolescent cohorts into two time points namely, immediately before (PND 23–27) and immediately after (PND 31–49) the adolescent period. Patwell et al. (2011) reported that early pre-adolescent (PND 23-27) fear conditioned mice exhibited complete contextual fear identical to that exhibited by the adult cohort. The contextual fear was subdued upon transition into adolescence (PND 29), whereas proceeding out of the adolescent period (PND 39) resulted in a re-emergence of the contextual fear.

Pattwell et al. (2011) thus suggests a period of suppression prior to and following adolescence (PND 29). During this interlude the context in which the fear conditioning is presented, is relayed as a cue and constructed as a fear memory. This period of suppression correlates to Fanselow's theory that states that "the contextual representation of the conditioning environment must be relayed to the affective system as a fear-signifying cue after, and only after, such a representation has been constructed by the cognitive system" (Fanselow, 1994). Furthermore, the period of suppression of contextual fear, as noted in pre-adolescent and adult mice (Pattwell et al., 2011), is consistent with previous findings (Chen et al., 2005; Raineke et al., 2010). Basically the interlude implies that the context in which the fear conditioning was presented was unable to elicit a fear response until the mice reached adulthood. This brief suppression can be viewed as an evolutionary, compensatory/survival mechanism. As increased contextual fear would be maladaptive to exploratory behaviour in the pre-adolescent rat; the time period in which rodents explore their surrounds, become independent and ultimately leave the nest (Spear & Brake, 1983). This study therefore poses an interesting concept in that fear memories formed during this suppression period remain intact and may be retrieved at a later stage,

i.e. adulthood. Replication of this study is important to better elucidate the inherent mechanisms involved in suppressing unwanted fear memories.

Even though like adolescents, the adult rats in our study showed strong contextual fear conditioning, they demonstrated a greater incidence of freezing in both the exposure to tone and exposure to context tests. The adolescent and adult rats of the present study exhibited similar patterns of fear extinction, which is extinction leading to a decrease in the fear conditioned response of rats. The latter finding concurs with the findings of a study by Hefner and Holmes (2007) that reported no age differences with regards to fear extinction. The ontogeny of contextual fear conditioning is of interest because it can provide a window to the ontogeny of encoding and memory organization (Brasser & Spear, 2004; Lariviere et al., 1990) and because it has been used as a marker for the development of brain function important for memory (Pugh & Rudy, 1996; Rudy & Morledge, 1994).

6.4.3 Summary of Behavioural Results

In summary, rats that received DCS followed by extinction training exhibited significantly less freezing than rats given saline. Specifically in the open field and light/dark box tests, pre-extinction DCS infusions decreased the fear response to below the baseline freezing time. The data demonstrated that intrahippocampal DCS infusion prior to extinction training facilitated extinction of conditioned fear responses. Furthermore, we demonstrated that DCS, at a dosage of 15mg/kg, may alleviate anxiety and depression-like behaviours. Overall, the present results support the administration of DCS as part of a treatment strategy to reverse PTSD-like symptoms. However we do acknowledge that more studies are needed in this regard to fully understand the impact of early

developmental trauma and successfully manage PTSD (Heim & Nemeroff, 2001; Coplan et al., 1996).

Table 6.1: Mean freezing time (seconds) of PND 33 and PND 60 rats in response to exposure of the context (fear conditioning). * $p < 0.05$

Group (n=30/group)	PND 33 freezing * (seconds)	PND 60 freezing* (seconds)
CS	15.10 (1.95)	29.94 (2.18)
SS	17.00 (2.14)	32.75 (1.55)
CD	14.96 (2.07)	29.12 (1.76)
SD	16.65 (1.93)	31.80 (1.21)

Table 6.2: Mean freezing time (seconds) with SEM of PND 33 and PND 60 rats in response to re-exposure of the context (associative fear memory). * $p < 0.05$

Group (n=30/group)	PND 33 freezing * (seconds)	PND 60 freezing * (seconds)
CS	5.12 (0.85)	42.13 (13.49) **
SS	6.50 (1.02)	43.93 (13.49) ***
CD	3.52 (0.99) **	19.27 (8.53) **
SD	2.00 (1.09) **	24.03 (14.95) ***

Table 6.3: Mean freezing time (seconds) in response to tone with SEM of PND 33 and PND 60 rats. * $p < 0.05$

Group (n=30/group)	PND 33 tone freezing (seconds)	PND 60 tone freezing (seconds)
CS	13.38 (0.54) *	52.23 (2.53) *
SS	20.22 (1.22) *	55.90 (2.89) **
CD	11.26 (0.44)	29.33 (1.95) *
SD	9.56 (0.53)	30.40 (3.26) **

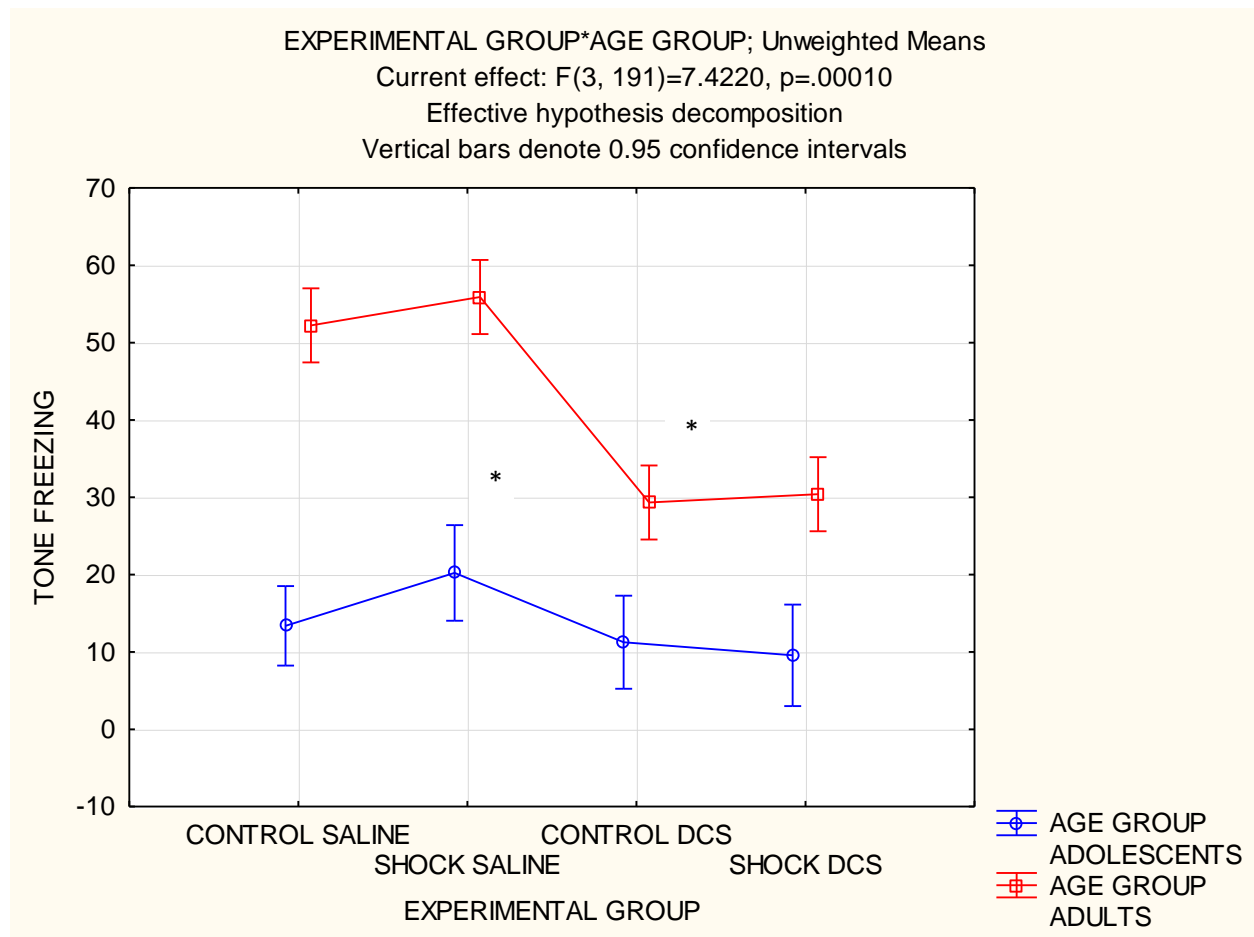


Figure 6.1: A line graph depicting time spent freezing while exposed to neutral tone in control, shocked and DCS treated animals of PND 33 and PND 60. DCS – D-cycloserine. Values are expressed as mean \pm SEM ($n=30/\text{group}$) in time (seconds). * $p \leq 0.05$

Adolescent fear conditioned rats (SS) displayed significant higher freezing times compared to their controls (CS) upon exposure to a tone ($p < 0.05$ Bonferroni Multiple comparison test). DCS treated adolescent rats exhibited freezing times similar to controls. Adult rats displayed a similar pattern of freezing behaviour, with a non-significant increase in freezing behaviour in shocked rats. DCS treated adult rats exhibited significantly lower freezing times compared to their controls ($p < 0.05$ Bonferroni Multiple comparison test).

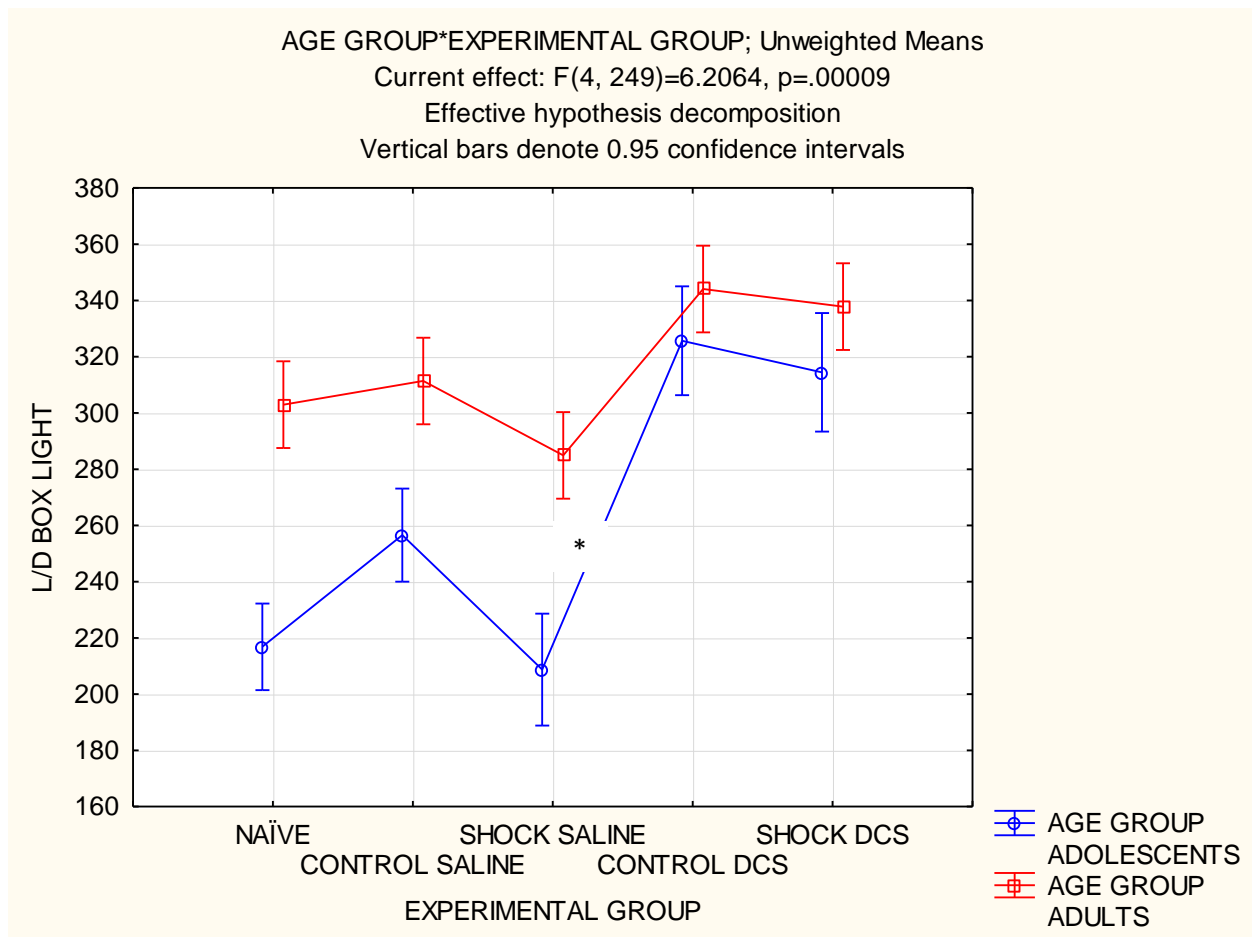


Figure 6.2: A line graph depicting the time spent in the light compartment of the light/dark box in naïve, control, shocked and DCS treated animals of PND 33 and PND 60. DCS – D-cycloserine. Values are expressed as mean \pm SEM (n=30/group) in time (seconds). * $p\leq 0.05$

Adolescent saline treated (CS and SS) and naïve animals spent significantly less time in the light compartment of the light/dark box compared to DCS treated rats (CD and SD) ($p < 0.05$ Bonferroni Multiple comparison test). A similar yet less pronounced pattern of time spent in the light compartment was displayed by adult animals, with DCS treated animals spending less time in the light compartment of the light/dark box.

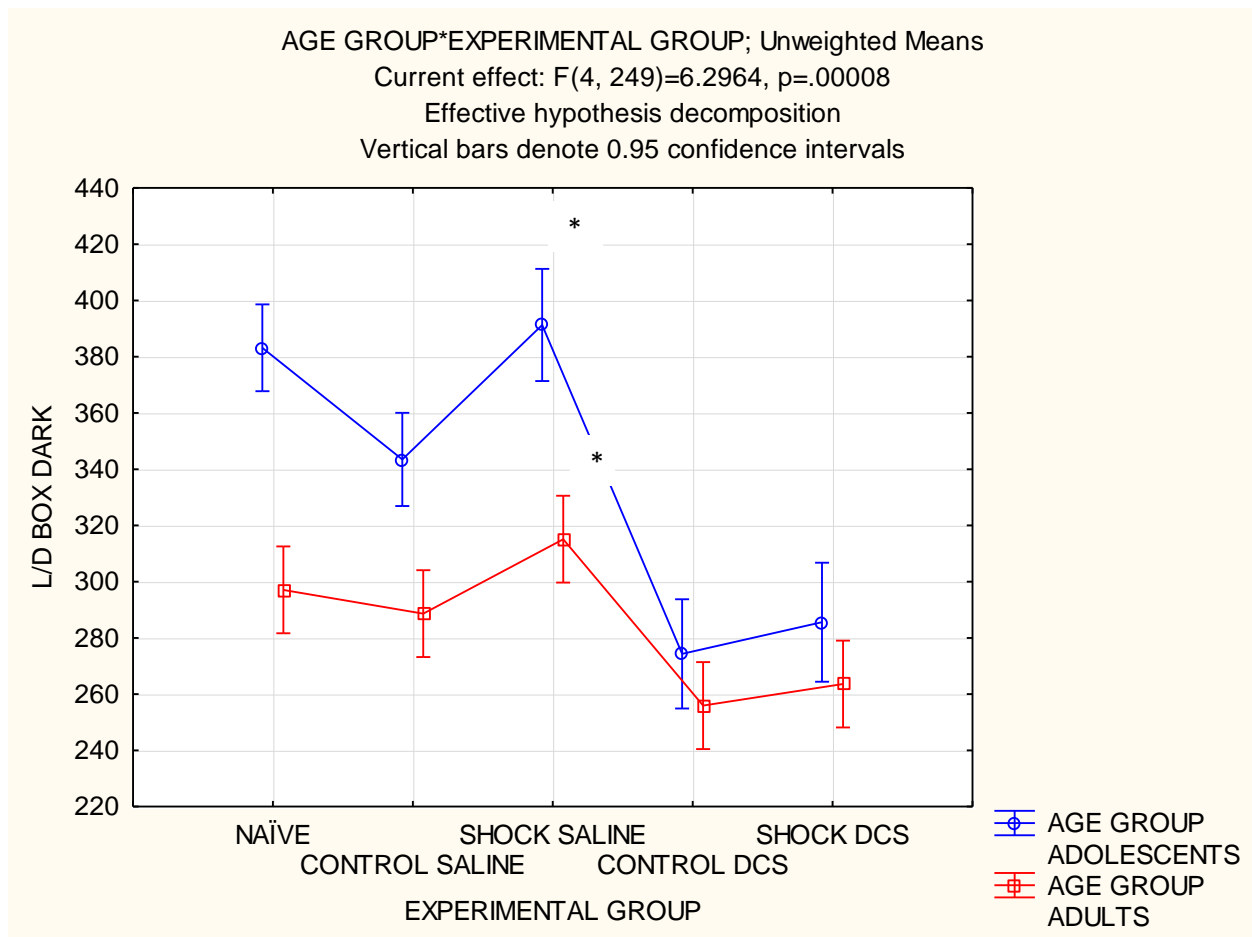


Figure 6.3: A line graph depicting the time spent in the dark compartment of the light/dark box in naïve, control, shocked and DCS treated animals of PND 33 and PND 60. DCS – D-cycloserine. Values are expressed as mean±SEM (n=30/group) in time (seconds). * $p \leq 0.05$

Both adolescent and adult saline treated (CS and SS) and naive animals spent more time in the dark compartment of the light/dark box compared to DCS treated rats (CD and SD), with fear conditioned (SS) rats of both ages spending the greatest amount of time in the dark compartment of the light/dark box ($p < 0.05$ Bonferroni Multiple comparison test).

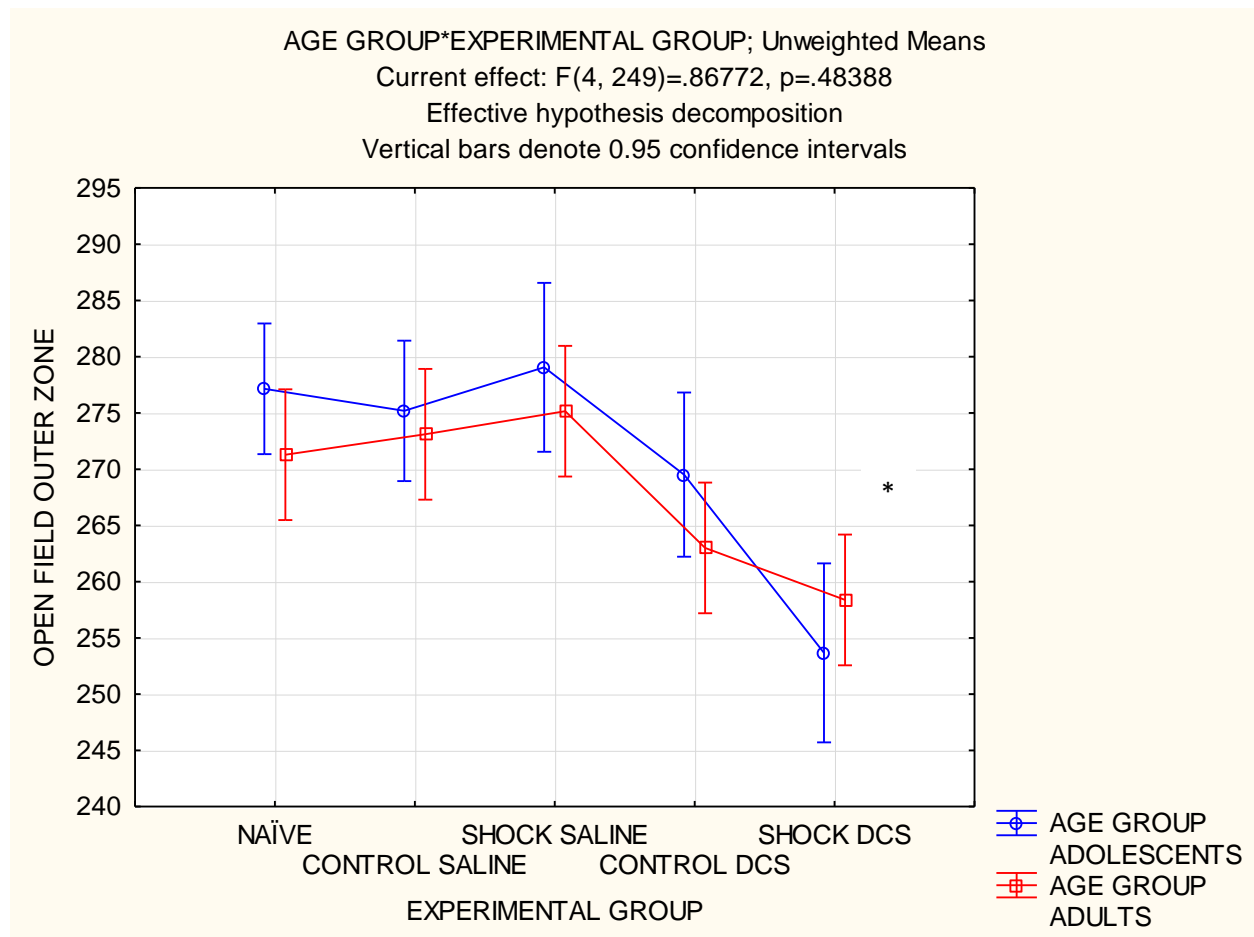


Figure 6.4: A line graph depicting the time spent in the outer zone of the open field in naïve, control, shocked and DCS treated animals of PND 33 and PND 60. DCS – D-cycloserine. Values are expressed as mean \pm SEM (n=30/group) in time (seconds). * $p\leq 0.05$

Both adolescent and adult DCS treated animal spent less time in the outer zone of the open field compared to their controls. DCS treated shocked rats spent significantly lower time in the outer zone of the open field ($p < 0.05$ Bonferroni Multiple comparison test). DCS treated shocked rats (SD) spent less time in the outer zone when compared to saline treated shocked animals (SS) ($p < 0.05$ Bonferroni Multiple comparison test).

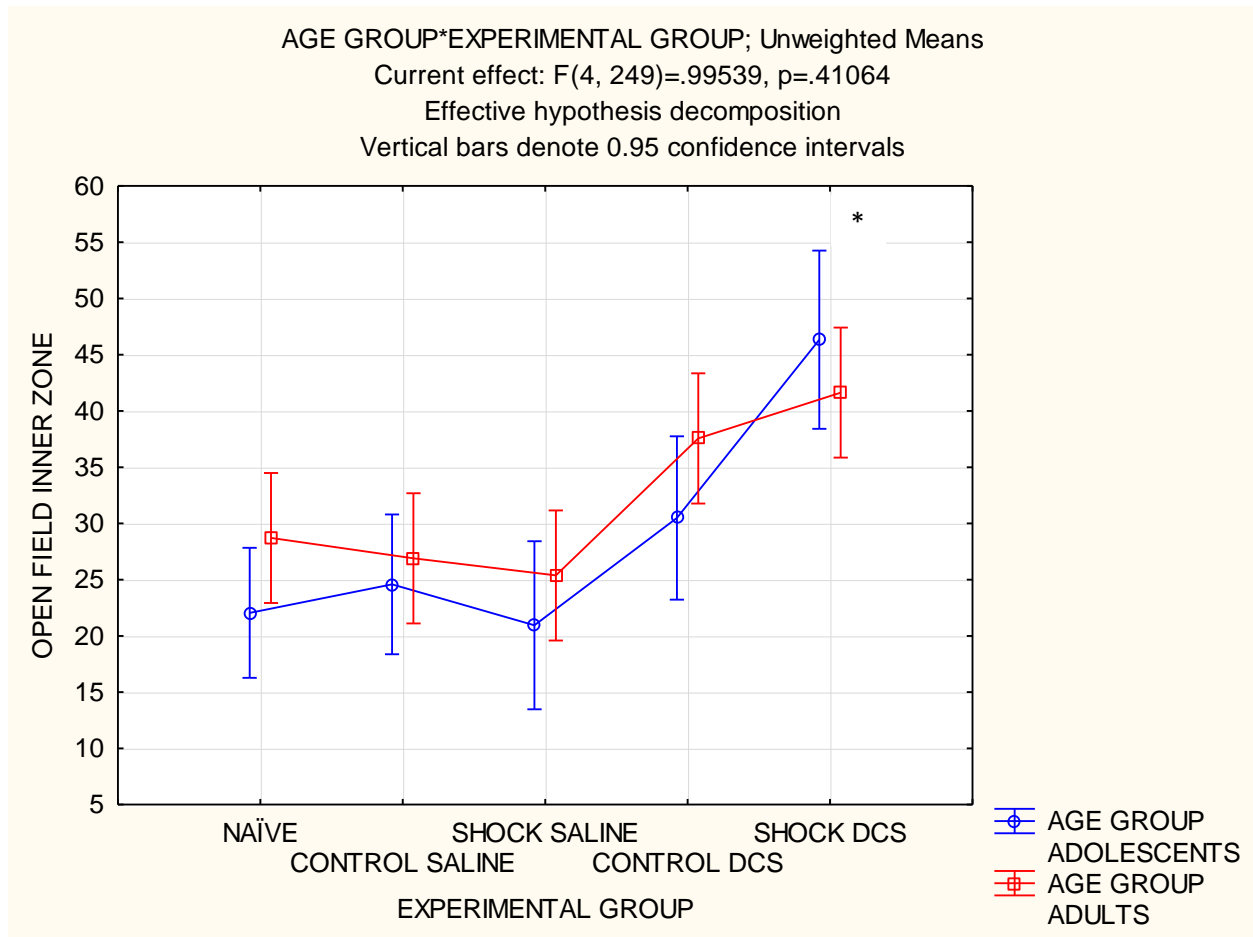


Figure 6.5: A line graph depicting the time spent in the inner zone of the open field test in naïve, control shocked and DCS treated animals of PND 33 and PND 60. DCS – D-cycloserine. Values are expressed as mean±SEM (n=30/group) in time (seconds).* p<0.05

Both adolescent and adult DCS treated animal spent significantly more time in the inner zone of the open field compared to the naïve and their controls counterparts (p<0.05 Bonferroni Multiple comparison test).

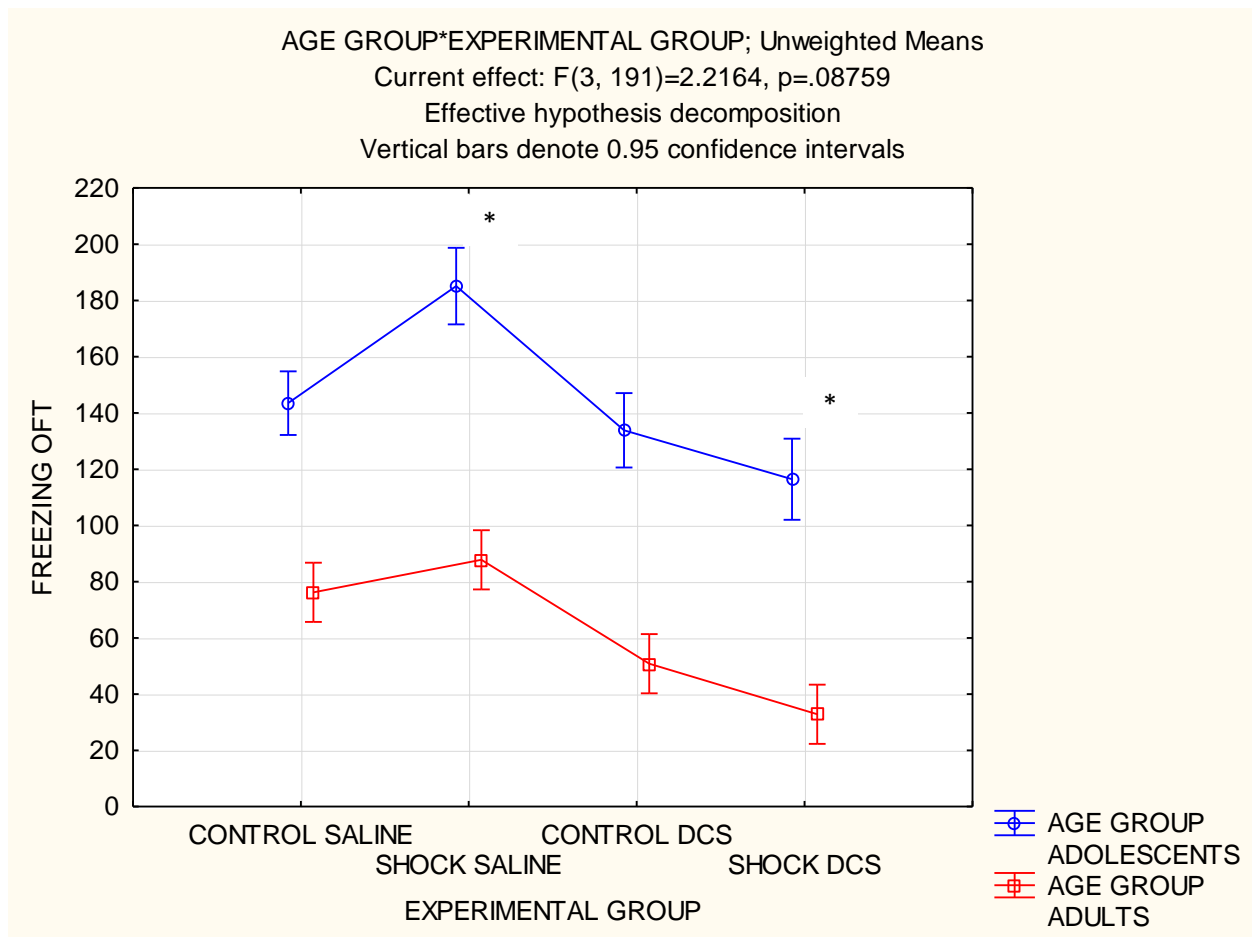


Figure 6.6: A line graph depicting the mean freezing time spent in the open field test in naïve, control shocked and DCS treated animals of PND 33 and PND 60. DCS – D-cycloserine. Values are expressed as mean \pm SEM ($n=30$ /group) in time (seconds). * $p\leq 0.05$

Fear conditioned (SS) adolescent and adult rats froze significantly more in the open field compared to their controls (SS) ($p < 0.05$ Bonferroni Multiple comparison test). DCS treated shocked (SD) adolescent animals displayed significantly lower freezing in the open field compared to saline treated shocked animals (SS) ($p < 0.05$ Bonferroni Multiple comparison test). The adult rats displayed similar patterns, but they froze to a lesser degree than the adolescents.

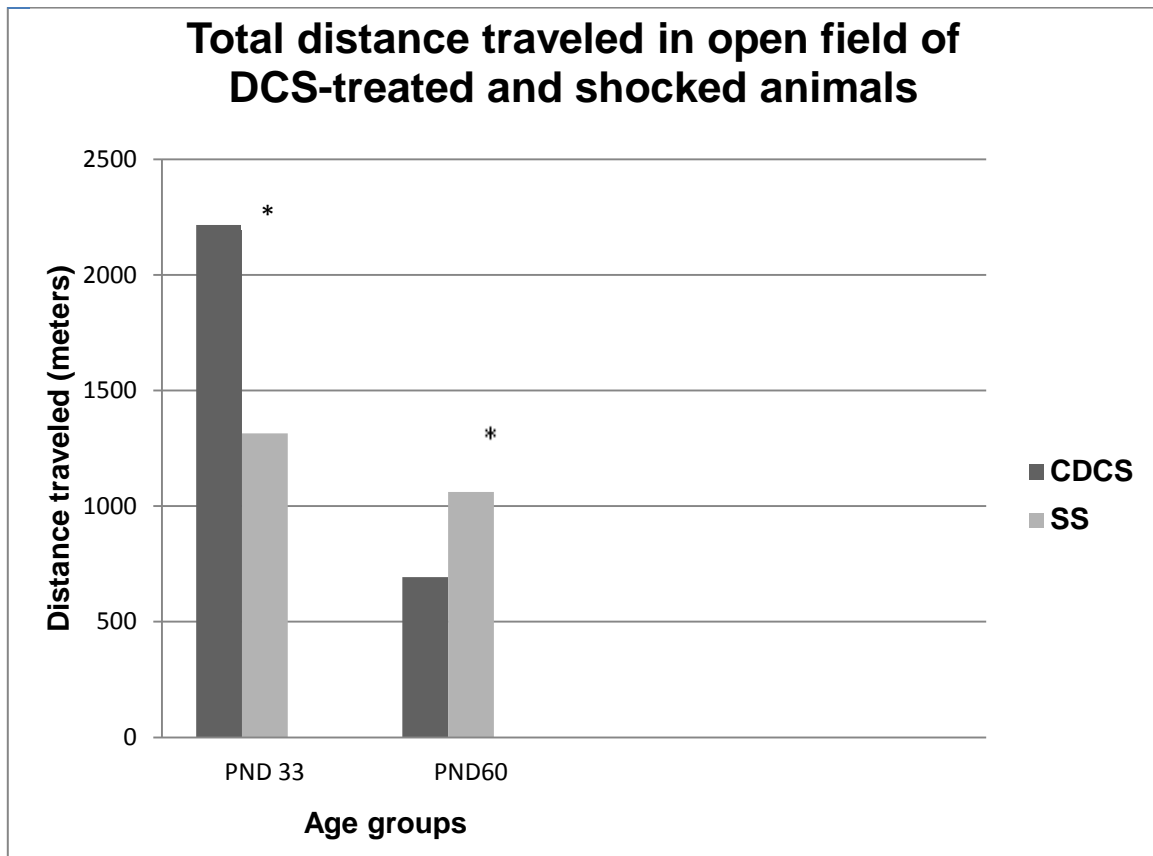


Figure 6.7: Histograms depicting the total distance traveled in the open field test in DCS treated and shocked animals of PND 33 and PND 60. CDCS – control+DCS, SS – shock+saline. Values are expressed as mean \pm SEM (n=30/group) in meters. * $p \leq 0.05$

Fear conditioned (SS) adolescent rats travelled significantly less in the open field compared to the DCS-treated rats (CDCS) ($p < 0.05$ Bonferroni Multiple comparison test). In contrast, the adult fear conditioned rats exhibited significantly greater locomotor activity compared to the DCS-treated rats ($p < 0.05$ Bonferroni Multiple comparison test).

Table 6.4: Mean duration of immobility (seconds) with SEM of PND 33 and PND 60 rats in forced swim test

Group (n=30/group)	PND 33 Immobility (seconds)	PND 60 Immobility (seconds)
Naïve	130 (11.31)	180 (15.93)
CS	135 (10.37) *	178 (13.83) *
SS	160 (7.57)	195 (8.02)
CD	110 (2.91)	154 (6.60)
SD	105 (3.37) *	142 (7.41) *

Table 6.5: Mean climbing attempts with SEM of PND 33 and PND 60 rats in forced swim test

Group (n=30/group)	PND 33 Climbing attempts	PND 60 Climbing attempts
Naïve	10 (1.18)	6 (0.83)
CS	12 (2.05)	7 (0.84)
SS	15 (1.59)	9 (1.12)
CD	7 (1.00)	3 (0.45)
SD	5 (0.68)	2 (0.48)

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Chapter 7

Neurochemical assessment of rats subjected to fear conditioning and DCS treatment

7.1 Introduction

To study the neurobiology of PTSD adolescent and adult rats were subjected to foot shocks in a certain context. This approach elicited PTSD-like symptoms in the shocked animals (see Chapter 5 for more detail), thereby rendering the current rodent model suitable for further investigation. The DHC is richly endowed with NMDARs and these receptors are greatly implicated in the encoding of contextual fear as well as fear extinction (Falls et al., 1992; Walker et al., 2002; Szapiro et al., 2003; Ledgerwood et al., 2003, 2004, 2005; Barad, 2005; Garakani et al., 2006). We subsequently assessed the molecular mechanisms by which the foot shocks induced PTSD-like symptoms and how DCS may act to reduce these symptoms. The study specifically focused on the NMDAR1 concentrations, considering that it represents the binding site for DCS (Sheinin et al., 2001). Considering that fear learning has also been shown to require signalling pathways involving nNOS and BDNF (Lu et al., 1999; Arancio et al., 2001; Puzzo et al., 2006; Resstel et al., 2008), we decided to measure nNOS and BDNF concentrations as well. The subsequent protein changes and signalling pathways are described in the next chapter (see Chapter 8 for more detail).

7.2 Methodology

Adult male Sprague-Dawley rats were subjected to foot shocks, half of which also received intrahippocampal infusions of DCS (Shock + DCS) and the other half saline (Shock +

Saline). Animals placed in the shock chamber receiving no shocks, but either saline (Control + Saline) or DCS (Control + DCS) infusions, served as controls. Upon completion of the experiments and behavioural assessments (see Section 4.5 in Chapter 4 for more detail), animals were decapitated on PND 40 (adolescent) and PND 67 (adulthood) respectively. We harvested the dorsal hippocampi of 10 adolescents and 10 adults (from each experimental group) for neurochemical analysis, as this brain region was not only the site of interest from an NMDA perspective, but also where the intrahippocampal DCS infusions were targeted. Commercially available ELISA kits were used for measuring NMDAR1, BDNF and nNOS concentrations (see Section 4.5 in Chapter 4 for more detail).

7.2.1 Statistical analysis

Statistical analyses were done with the assistance of the Centre for Statistical Consultation of the University of Stellenbosch. The software package Statistica was used to perform statistical tests. Shapiro-Wilk normality test indicated that our behavioural data had a Gaussian distribution. Subsequently the biochemical parameters were analyzed using a one-way ANOVA test that was followed by the Bonferroni *post-hoc* test where appropriate. The level of significance was accepted as $p < 0.05$. Data is reported as pg/mg wet weight.

7.3 Results

7.3.1 Fear conditioning alters NMDAR1 expression

A significant difference was noted in NMDAR1 levels after exposure to the aversive stimulus in both age groups studied (Figure 7.1). While control+saline treated animals had NMDAR1 levels similar to naïve rats, exposure of animals to footshock led to a significant increase in NMDAR1 concentrations (Figure 7.1; $p < 0.05/p = 0.00$, Bonferroni's

Multiplecomparison test). This increase was significantly reduced in the group of shocked animals that received DCS infusions (Figure 7.1; $p < 0.05/p = 0.00$, Bonferroni's Multiple comparison test). Control animals that only received DCS treatment also had significantly lower NMDAR1 levels than their saline-treated counterparts (Figure 7.1; $p < 0.05/p = 0.00$, Bonferroni's Multiple comparison test). There was however no significant difference in NMDAR1 concentrations between PND 33 and PND 60 rats in all the respective groups (Figure 7.1; $p < 0.05$, Bonferroni's Multiple Comparison test; $F = 4.53$, $p = 0.13$).

7.3.2 Fear conditioning alters nNOS expression

Interestingly, nNOS levels followed a similar pattern to the NMDAR1 levels expressed in both age groups studied. In both adolescent and adult rats a significant difference was noted in nNOS levels after exposure to the footshock, with the fear conditioned animals expressing greater nNOS levels when compared to their respective controls (Figure 7.2; $p = 0.00$, Bonferroni's Multiple comparison test). Again treating the animals with DCS significantly decreased the foot shock-induced elevation in nNOS levels (Figure 7.2; $p = 0.00$, Bonferroni's Multiple comparison test). However a significant difference in nNOS expression levels between the two age groups was noted, with adults expressing significantly higher nNOS levels compared to adolescents (Figure 7.2; $p = 0.00$, Bonferroni's Multiple comparison test).

7.3.3 Fear conditioning alters BDNF expression

A significant difference was noted in BDNF levels when comparing all experimental groups at both age levels. In contrast to NMDAR1 and nNOS levels, adolescent and adult fear conditioned animals displayed a significant decrease in BDNF levels when compared to

their controls (Figure 7.3; $p=0.02$, Bonferroni's Multiple comparison test). In animals that were treated with DCS, the BDNF concentration was significantly higher than the saline-treated controls for both shocked and non-shocked groups (Figure 7.3, $p = 0.02$, Bonferroni's Multiple comparison test). A significant difference between the age groups was also noted for BDNF expression levels, with adults expressing significantly lower BDNF levels compared to adolescents (Figure 7.3; $p =0.02$).

7.3.4 Tabled summary of neurochemical results

Neurochemical Parameters	Fear conditioning (footshock)	DCS treatment
NMDAR-1	↑	↓
nNOS	↑	↓
BDNF	↓	↑

7.4 Discussion

It has been postulated that specific neurochemical substrates mediate the dysfunctional stress response that underlies acquired anxiety and PTSD (Yehuda & McFarlane 1995). Impaired learning and memory processes have also been implicated in PTSD (Friedman, 1997; Vermetten & Bremner, 2002) and fear conditioning. Furthermore, neuronal excitability has been shown to underlie learning and memory tasks (Disterhoft et al., 1986; Disterhoft & Oh, 2006). However, before the fear conditioned-induced neurochemical effects and their role in these experience-dependent forms of neural plasticity can be discussed, an understanding of the physiological neuronal response mechanisms crucial for learning and information storage is necessitated.

7.4.1 The role of NMDAR, nNOS and BDNF in fear conditioning

The HC is important for learning and memory. Specifically, the DHC has an important role in contextual fear conditioning (McEchron et al., 1998; Chowdhury et al., 2005; Misane et al., 2005). Moreover, studies have shown that experience-dependent forms of learning and memory are NMDAR-dependent (Bear & Malenka, 1994; Davis et al., 2006). Under normal, physiological conditions the hippocampal NMDARs are activated by the simultaneous depolarization and co-binding of glutamate and glycine to the NMDAR2 and NMDAR1 subunits, respectively (Johnson & Ascher, 1987; Kleckne & Dingledine, 1988). Upon binding of the co-activators, the NMDAR channel opens up, allowing a transient influx of Ca^{2+} into the post synaptic cell which activates various intracellular signalling cascades, and results in neurotransmission. Neuronal excitability reduces the post-burst afterhyperpolarization (AHP) - which serves as a limiting factor to subsequent excitations (Lancaster & Adams, 1986; Storm, 1990; Sah & Bekkers, 1996) and prevents overexcitation of the post synaptic membrane and promotes increased information storage (Sah & Bekkers, 1996). These electrical processes facilitate long-lasting changes in hippocampal synaptic strength, which has been associated with LTP (Kato et al., 1998). Thus hippocampal LTP has been proposed as a cellular model of learning and memory due to the notion that memories are encoded by the modification of synaptic strength (Bliss & Collingridge, 1993).

Synaptic strength is further reinforced by glutamatergic neurotransmission via the NMDAR with subsequent Ca^{2+} influx binding to calmodulin. This calcium/calmodulin complex activates the nNOS enzyme to produce NO (Garthwaite & Boulton, 1995). Interestingly, NO is a gas that has been postulated to be central to LTP and memory formation (Lu et al. 1999; Arancio et al. 2001; Puzzo et al., 2006). In particular, acting as a retrograde

messenger (Snyder 1992) NO enhances presynaptic glutamate release and so strengthens the synaptic transmission. NO is known to have both neuroprotective (Melino et al., 1997; Ogura et al., 1997, So et al., 1998) and neurodamaging effects; depending on the quantity expressed. Under physiological conditions however, small and highly regulated bursts of NO is produced to reinforce LTP (Garthwaite & Boulton, 1995).

Another important downstream target of glutamatergic NMDAR neurotransmission is BDNF. This neurotrophic factor has been identified as a neuromodulator of LTP. For example, during physiological learning Ca^{2+} -binding leads to the phosphorylation of the nuclear activator protein cyclic adenosine monophosphate (cAMP) Response Element Binding Protein (CREB), resulting in BDNF transcription. This nerve growth factor increases the number of synapses (Figurov et al., 1996) and changes neuronal morphology (McAllister et al., 1997) which ultimately mediates long-lasting changes in synaptic plasticity. Interestingly, NO was also found to directly modulate BDNF expression (Xiong et al., 1999). It should therefore be noted that the neurochemical parameters of interest in this study, have dichotomous roles. Under physiological conditions, they not only subserve learning and memory, but also promote beneficial changes in the brain. However, under pathological conditions these neurochemical parameters may be damaging to the neurons.

7.4.2 Fear learning and memory

PTSD development is associated with learned fear conditioned responses which serve as a reminder of the traumatic event by presenting as intrusive memories (Kenny et al., 2009). Emotional memory tends to be long-lasting and has been identified as an important regulator of behavioural responses in humans and animals. Because it is essential for

survival, the memory of fear is easily established by immediate transferral from short-term to long-term memory. This consolidation rate is based upon many factors, such as emotional state and association. Given the aforementioned, memories are usually filed along with the context in which they were learned; underscoring the role of the HC in associative learning (Martin et al., 2000; Johnson et al., 2012) and mediation of conditioned fear responses (Alonso et al., 2005).

Considering that fear conditioning is commonly used to study PTSD memory processes in rodents (Kamprath & Wotjak, 2004, Siegmund & Wotjak, 2006; 2007a), we employed a contextual fear conditioning model which incorporated associative learning and memory processes (Siegmund & Wotjak, 2007). The current contextual fear conditioning model consisted of applying a foot shock, in a certain context, to elicit a fear response. We measured the conditioned fear response as freezing time (Fendt & Fanselow, 1999; Davis, 2000; Nemeroff et al., 2006; Siegmund & Wotjak, 2006; Yehuda & Le Doux, 2007) and found the fear conditioned rats exhibited greater freezing immobility compared to their controls. The increased freezing was in response to both the aversive footshock as well as upon re-exposure to the shock box (Blanchard & Blanchard, 1969; Fanselow, 1980; Le Doux et al., 1998); suggesting that learning occurred during the contextual fear conditioning process. To confirm whether learning and memory consolidation took place, NMDAR1 levels were measured in the DHC of fear conditioned rats. The rationale for measuring NMDAR subunit involvement, in learning and memory, was based on restricted genetic deletion NMDAR1 studies of the HC which found impaired spatial memory and NMDA-dependent LTP (Tsien et al., 1996 a, b).

7.4.2.1 The role of NMDAR1 in fear learning and memory

In the current study trauma, experienced as electric footshock, evoked a significant increase in NMDAR1 levels as demonstrated in the fear conditioned (shock + saline) rats versus their controls. This finding is in alignment with animal experiments of fear and stress which demonstrated increased NMDAR1 expression (Fitzgerald et al., 1996), altered function and binding of the NMDAR glycine site (Yoneda et al., 1994), as well as augmentation of glutamate release (Bagley & Moghaddam, 1997; Ho et al., 2000) in various brain areas such as the HC, ventral tegmental area and prefrontal cortex. In addition, our findings correlated well with those reported in the study by Yamamoto et al. (2008) who utilized a single bout of prolonged stress as trauma and demonstrated a significant upregulation of NMDAR mRNA levels. Furthermore overstimulation of NMDARs has been observed in PTSD patients and has been suggested to be responsible for the neurodegeneration and neuropathological features observed in this disorder. This notion therefore explains the behaviour of our shock-exposed rats such as increased freezing response and anxiety-like behaviours.

Freezing time is indicative of the conditioned fear response to a traumatic stimulus (Fench & Fanselow, 1999; Davis, 2000; Nemeroff et al., 2006; Siegmund & Wotjak, 2007; Yehuda & Le Doux, 2007). In the current study, the traumatic stimulus (i.e. footshock) elicited a significant increase in both the conditioned fear response (i.e. freezing to context) and NMDAR1 levels in the fear conditioned rats. Our findings correlate with previous learning and memory studies which demonstrated that LTP induction necessitates activation of the glutamatergic NMDAR system (Morris et al., 1986; Lanthorn 1993, Quartermain et al., 1994; Sakimura et al., 1995; Tsien et al., 1996) and intrinsic modifications of neuronal excitability subserve learning-related behaviours (Saar & Barkai, 2003; Zhang & Linden,

2003; Disterhoft et al., 2004). Moreover, trauma/fear studies corroborated that long-lasting negative behavioural effects, triggered by traumatic events, are established upon activation of NMDARs and their subsequent signaling pathways (Walker et al., 2002; Ledgerwood et al., 2005; Sziray et al., 2006). Specifically, phosphorylation of the essential NMDAR1 was demonstrated to regulate NMDAR activity. This NMDAR subunit is therefore used as an indicator of NMDAR functionality.

Considering the above, we elucidate on the role of increased NMDAR1 levels in the context-shock association learning exhibited by the fear conditioned rats. In 1992, Bell and colleagues originally proposed the neural sensitization theory based in part on evidence showing increased hippocampal long-term synaptic sensitivity in response to previous chemical and electrical stimulation i.e. LTP. Since then several other researchers have expanded on this theory (Bell et al., 1992; Friedman, 1994; Sorg & Prasad, 1997; Bell et al., 1999; Sorg, 1999). We therefore proposed that repeated activation of the NMDARs in our study resulted in a perpetual state of hyperexcitability i.e. the receptors become more sensitized and responsive to subsequent inputs. The footshock can be regarded as an acute, high-frequency sensory input that results in prolonged firing of the action potential. In turn, excessive glutamate is released (Kato et al., 1998), overactivating the NMDARs and causing a higher Ca^{2+} influx than found in physiological conditions. The result is a greater amplification of the downstream intracellular cascades which results in a tremendous increase in the potency of the initial signal. We suggest that the hippocampal neurons became less inhibited and more easily “turned on” by the excessive binding of glutamate to the NMDAR. The repeated use of the NMDARs increases the efficiency of their synaptic connections. Supporting our theory are various studies emphasizing the importance of plasticity in learning and memory (Zhang & Linden, 2003). Our study illustrated that synaptic potentiation sensitization in animals with a greater number of

overactivated NMDARs, as observed in the fear conditioned rats, learned more readily than their controls.

We also suggest that similar to physiological experience-dependent learning, the post-burst AHP mechanism may facilitate greater storage of the fear memories acquired during the context-shock association by means of the amplified LTP (Sah & Bekkers, 1996). Thus, animals with a greater number of overactivated NMDARs, as observed in the fear conditioned rats, retain the fear memory better. The observed neurochemical changes within NMDARs can be corroborated by the behavioural results that indicate increased freezing upon re-exposure to the shock box, which served as a reminder of the traumatic event (i.e. associative fear response). Several additional animal conditioning (de Jonge et al., 1990; Moyer et al., 1996, 2000; Kuo, 2004) and learning (Oh et al., 2003; Tombaugh et al., 2005; Ohno et al., 2006b) experiments have illustrated a learning-induced reduction in AHP which persisted for days after training was completed (Moyer et al., 1996; Thompson et al., 1996; Saar & Barkai, 2003; Zelcer et al., 2006). These findings demonstrated an inverse relationship between learning and the AHP mechanism which resulted in LTM.

Furthermore, we propose that the sensitized NMDARs can account for the non-associative freezing response. The overstimulated NMDARs become more responsive and as such presentation of an unexpected loud tone, during the non-associative fear test, elicited greater freezing times in fear conditioned rats versus their controls. Thus the hypersensitized NMDARs manifested in a hyperaroused behavioural phenotype. The findings of the present study correlate to the clinical setting, as PTSD patients were shown to present with overactive NMDARs as well as hyperarousal symptoms when presented with non-specific cues (DSM-IV-TR, APA 2000).

Overall, increased NMDAR1 levels here indicate a role for dorsal hippocampal NMDARs in associative learning and memory mechanisms during the development of conditioned fear responses. Furthermore, we recommend that sensitized NMDARs underlie the associative and non-associative behavioural fear responses exhibited by our fear conditioned rats. Therefore, we conclude that during fear conditioned learning, cellular processes similar to those utilized in physiological LTP, were stimulated. The only difference being, that the stimulation (footshock) contributing to the fear conditioned learning was of greater magnitude compared to physiological conditions.

As with physiological learning and memory processes, our fear conditioned learning paradigm also required NMDAR activation. However, opposite to the beneficial effects of physiological activation of NMDARs, an overstimulation of NMDARs is known to cause neuronal loss. Since the presence of neuronal cell loss in the current experiment was not assessed, this explanation remains speculative. Also, the exact mechanism how foot shocks can lead to increased protein expression (i.e. increased NMDAR1 levels) in the post synaptic membrane was beyond the scope of the present study and will have to be investigated further. Our study subsequently focused on some downstream intracellular targets of the NMDAR in an attempt to understand how increased NMDAR expression may come about during fear conditioning. Since BDNF and nNO have also been implicated in LTP, we determined the levels of nNOS and BDNF in the DHC of our rats.

7.4.2.2 The role of nNOS in fear learning and memory

Based on the studies by Garthwaite and colleagues, who demonstrated that glutamatergic NMDAR activation stimulates nNOS production (Garthwaite et al., 1988; Garthwaite

&Bouton, 1995), we proposed that nNOS levels would mirror our NMDAR findings. We indeed observed a similar pattern of expression for nNOS as for NMDAR1 levels. Fear conditioned animals exhibited increased levels of nNOS levels in response to the aversive stimulus of the present study. This finding corresponds with an animal model of trauma utilizing TDS, which resulted in increased hippocampal nNOS activity (Oosthuizen et al., 2009).

As activation of the NMDARs lead to a Ca^{2+} influx, it activates nNOS which in turn produces NO (Garthwaite &Boulton, 1995). We therefore propose that the observed increase in NMDAR1 levels of our fear conditioned rats directly stimulates an increase in nNOS levels in the fear conditioned rats. As evidenced by our synaptic potentiation sensitization theory for NMDARs in fear conditioned learning, we suggest that nNOS also underlies this form of LTP. Previous findings, in support of our concept, demonstrated that both nNOS and its product NO, are implicated in synaptic plasticity (Lu et al., 1999; Arancio et al., 2001; Puzzo et al., 2006). In particular, rat studies using pharmacological site-specific NO blockade, showed impaired fear learning (Schafe et al., 2005; Resstel et al., 2008). Knockout mice studies have further confirmed a role for the nNOS gene and NO signaling in fear learning; with knockout mice exhibiting impaired contextual fear learning.

The stress-restress study of Oosthuizen et al. (2003) confirmed an interaction between NOS and NMDARs, specifically demonstrating that increased NOS results in hippocampal NMDAR changes. We therefore propose a positive feedback loop between the NMDARs and nNOS i.e. an overactivated NMDAR will increase nNOS levels which inturn will

stimulate and excite NMDARs to further enhance nNOS levels, ultimately resulting in greater hippocampal LTP.

In addition to its role in learning, we infer a role for nNOS in the late phase of LTP which involves memory consolidation as indicated by previous studies (Medina & Izquierdo, 1995; Rickard et al., 1998; Ota et al., 2008). We also observed increases in both nNOS levels and the behavioural associative fear response of fear conditioned rats to the shock box, which indicated that these animals remembered the previous aversive situation. It can therefore be concluded that the excessively increased hippocampal nNOS levels contribute to excessive consolidation of the original fear memory in the DHC of the fear conditioned rats. Collectively, these findings explain the repetitive intrusive memories that characterize PTSD. Moreover, these findings confirm the essential role of nNOS and NO in learning and memory (Bon & Garthwaite, 2003; Púzserová et al., 2006; Bernátová et al., 2007 b).

7.4.2.3. The role of BDNF in fear learning and memory

BDNF directly activates voltage-gated sodium channels in order to mediate rapid depolarization of target neurons (Blum et al., 2002) e.g. increasing NMDAR currents (Levine et al., 1998). Furthermore, an interaction between BDNF and glutamatergic receptors on synaptic plasticity has been demonstrated, whereby BDNF has been shown to heighten glutamate release, enhance ionotropic glutamate NMDAR activity and increase phosphorylation of NMDAR subunits (Minichiello, 2009). In a study employing a PTSD model to examine the impact of prior stress experience on subsequent contextual fear memory formation, it was shown that the earlier trauma resulted in enhanced freezing

levels that was associated with epigenetic changes to the BDNF gene in the HC (Takei et al., 2011). Collectively these observations point to a notable involvement of BDNF in PTSD, thereby justifying further investigation into the role of this neurotrophic factor in fear extinction.

BDNF plays a role in susceptibility to developing stress-related mood disorders. Studies have repeatedly shown that chronic restraint stress or unpredictable stress can lead to decreases in hippocampal mRNA and protein levels of BDNF in mice and rats (Duman & Monteggia, 2006). A growing body of research on BDNF also suggests a critical role for this neurotrophin in synaptic plasticity and memory (Lu et al., 2008; Korte et al., 1995; Mu et al., 1999). Numerous studies have shown BDNF to be expressed in glutamatergic neurons at both the pre-synaptic and post-synaptic sites (Minichiello, 2009 Review). The present study reported a significant reduction in BDNF expression in fear conditioned rats (shock + saline) compared to their controls. It is well documented, in both humans and animals, that various stressors alter hippocampal structure and ultimately function (Dell'Osso et al, 2009; Uys et al., 2006) via altered neurotrophin levels. Specifically, genetic studies have shown that the hippocampal BDNF gene is particularly susceptible to modulation by stress (Takei et al., 2011). Our finding of trauma-induced decreased BDNF levels corresponds to clinical studies demonstrating significantly lower BDNF levels in PTSD patients (Dell'Osso et al, 2009; Hauck et al., 2009, 2010).

Animal studies of stress have demonstrated decreased BDNF activity in the HC (Bazak et al., 2009; Lippmann et al., 2007; Nair et al., 2007; Nibuya et al., 1999; Rasmusson et al., 2002; Tsankova et al., 2006). Of these, preclinical footshock studies in particular reported decreased hippocampal BDNF expression (Rasmusson et al., 2002; Duman, 2002). A rat

study by Kozlovsky and colleagues (2007) is noteworthy as it demonstrated a correlation between PTSD-like behavioural responses and significantly down-regulated hippocampal BDNF mRNA in trauma-exposed rats. BDNF has also been implicated in LTP (Korte et al., 1995; Mu et al., 1999), with a loss of BDNF signalling leading to decreased LTP (Patterson et al., 1996; Monteggia et al., 2004) and impaired learning and memory (Lu et al., 2008), as observed in behavioural paradigms. Research on LTP has demonstrated suppression thereof in the dorsal CA1 hippocampal region in response to prolonged stress (Diamond et al., 2005; Joëls, 2008; Kim & Diamond, 2002). The CA1 region has also been recognized as the most sensitive site for stress-induced BDNF deoxyribonucleic acid (DNA) hypermethylation.

As BDNF has been implicated in neuron differentiation, survival and plasticity, we propose that decreased levels observed in our fear conditioned rats may hinder neurogenesis (Benraiss et al., 2001; Lee et al., 2001; Pencea et al., 2001; Kato-Semba et al., 2002), compromising the hippocampal neurons and result in poor maintenance of fear-related memories. Moreover, Heldt and colleagues (2007) investigated the effects of a hippocampal-specific BDNF gene deletion and found the lack thereof resulted in reduced learning as well as a reduction in fear extinction. These two findings of Heldt et al. (2007) are characteristic of the cognitive and emotional impairments exhibited by PTSD patients. Our initial finding that trauma induced a significant decrease in hippocampal BDNF levels of rats, confirms the essential role of this neurotrophin in fear memory formation, fear extinction and PTSD pathophysiology. Thus we propose that the decreased BDNF levels contribute towards the cell's vulnerability to subsequent stressors. Previous research has suggested a role for stress-modulation on the hippocampal BDNF gene, however the mechanisms whereby trauma affects this gene remains to be elucidated.

7.4.3 The effect of DCS treatment on the expression level of NMDAR, nNOS and BDNF in fear conditioning

Considering that the DHC and NMDARs are implicated in the modulation of fear extinction (Garakani et al., 2006; Falls et al., 1992), another aim of the present study was to examine the effects of pre-extinction intrahippocampal DCS infusion on the neurochemistry of this brain region. Although DCS readily crosses the blood-brain-barrier (D'Souza et al., 2000) when administered systemically, intrahippocampal infusion is the fastest way to deliver the drug and allows for direct examination of the drug's effects on these glutamatergic receptors in this limbic region.

The current study was specifically interested in the NMDAR1 considering that it is known as the glycine recognition site of the NMDAR to which DCS binds (Hollman et al., 1993). Furthermore, the interest in the NMDAR1 subunit was due to studies demonstrating its presence in all NMDARs as well as studies showing a lack of NMDAR1 results in dysfunctional NMDARs (Chazot, 2004). A difference exists between the immature and mature rat brain regarding the composition of NMDAR complexes i.e. the developing HC expresses NMDARs as diheteromers NR1/NR2B and triheteromers NR1/NR2B/D respectively (Pina-Crespo & Gibb, 2002) with the adult HC expressing NR2A and NR2B in pyramidal cells and NR2C subunits in different interneurons (Monyer et al., 1994). Activation of NMDARs necessitates the binding of glycine to NMDAR1 and binding of glutamate to NR2 subunits. Therefore we suggest that DCS, which binds to the glycine site of NMDAR, leads to the receptor's subunit 1 activation, subsequently triggering downstream messenger systems that may affect synaptic plasticity.

DCS treatment significantly decreased NMDAR1 levels in both shocked animals as well as controls. This finding corresponds to preclinical studies utilizing inhibitors of glutamate

reuptake which causes increased glutamate levels in the synaptic cleft and ultimately decreased NMDARs (Cebers et al., 1999). Similarly, our finding may reflect the possible neuroprotective effect of DCS on increased NMDAR activation and neurotoxicity following fear conditioning. Previous studies have demonstrated the counteractive effects of DCS on NMDAR overstimulation, acting as a possible neuroprotective mechanism (Naskar & Dreyer, 2001). Moreover, the SPS study of Yamamoto et al (2008) also utilized repeated administration of DCS which was found to eliminate the enhanced NMDAR mRNA levels noted in fear conditioned rats.

Fear extinction is believed to be a form of new learning. Pharmacological studies have shown that DCS enhances NMDAR activity by binding to the NMDAR1 site leading to activation of targets downstream of the receptor. Of these, BDNF has been well studied. In our study BDNF levels of DCS treated rats were significantly increased. This observation is in line with reports showing activation of the NMDAR leading to increased BDNF expression (Marini et al., 1998; Yaka et al., 2003). Furthermore, this finding suggests that DCS might act via BDNF and its downstream signalling pathways in enhancing fear extinction.

Several experimental studies have implicated BDNF in fear learning and memory (Chattwal et al., 2006; Rattiner et al., 2004 a, b). Specifically, BDNF signalling facilitates LTP via sustained TrkB activation (Kang & Schuman, 1996; Nagappan & Lu., 2005). Considering the essential role of BDNF in this form of synaptic plasticity (Korte et al., 2005), we hypothesize that the modulation of this neurotrophin by DCS during fear extinction might increase the growth and differentiation of dorsal hippocampal neurons and thus strengthen their connections resulting in greater enhancement of fear extinction.

Changes in BDNF levels, due to pre-extinction DCS treatment, mirrored the improvement of PTSD-like symptoms noted in the current behavioural results of DCS treated rats i.e. less freezing behaviour and anxiety-like symptoms.

7.4.4 Role of age

Studies have shown that the brain continues to develop throughout the adolescent period (Markus & Petit, 1987; Sowell et al., 1999) and that the enhanced vulnerability to trauma during adolescence is due to glutamatergic neurotransmission, in particular the NMDAR system. Further evidence has shown that NMDAR subunit expression is developmentally regulated during brain maturation (Watanabe et al., 1992, 1993; Jin et al., 1997; Wenzel et al., 1997; Barria & Manilow, 2002; Magnusson et al., 2002; Law et al., 2003; Chang et al., 2009). There were however, no significant differences in NMDAR1 expression between both age groups (for all the experimental groups). Our result is supported by the 2006 in situ hybridization study by Magnusson and colleagues, in which the authors observed no effect of age on NMDAR1 subunit mRNA densities in the dorsal hippocampus. Our finding is also in line with that of Hsiesh and colleagues (2002); little differences were noted between PND 25 and adult Sprague-Dawley rats regarding their expression of NMDAR1 subunit levels. A clinical study of the human HC further confirmed our finding i.e. they found no differences in NMDAR1 levels between adolescents and young adults (Law et al., 2003).

However, in our literature review we came across five studies implicating an age-related NMDAR hypofunction (Gonzales et al., 1991; Wenk et al., 1991; Magnusson & Cotman, 1993; Saransaari & Oja, 1995; Magnusson & Sammonds, 1998). In addition, animal studies ranging from macaques to rodents, have reported decreased NMDAR1 expression

in the dentate gyrus (Gazzeley et al., 1996), cortex and HC (Magnusson & Sammonds, 1998) of these aged animals with significant decreases in hippocampal NMDAR1 expression noted in mice over a time period of 10 to 30 months (Magnusson et al., 2002). Of note is the existence of discrepancies in the literature with regards to the effects of aging on hippocampal NMDAR expression. Differences in observations are likely to arise as a result of variations in type of stressors, duration of stress, choice of behavioural assay or endpoint parameters, rodent strain and brain pathway examined.

In the current study nNOS levels were decreased in adolescent rats compared to their adult counterparts. A study by Chalimonuik et al. (1998) demonstrated the effects of age on hippocampal NO synthesis and cyclic guanosine monophosphate (GMP) levels. Aging is characterized by memory impairment and an increase in nNOS as observed in our adult rats may be due to the neurotoxic effects of this enzyme. As activation of NMDARs subsequently lead to the activation of nNOS and numerous calcium dependent enzymes, this increase may have deleterious effects by resulting in cell death and neurodegeneration (Dawson & Dawson, 1995).

The developmental regulation of NMDAR subunit expression has been demonstrated to lead to changes in the functional and pharmacological properties of NMDARs. Several studies suggest that NMDARs influence synaptogenesis, neuronal growth and plasticity, as well as the fine-tuning of neuronal connections (Kloda et al., 2007). These properties mirror the functional properties of the neurotrophic factor BDNF. The adolescent and adult experimental groups exhibited a similar BDNF expression pattern. However adolescent rats displayed slightly higher BDNF levels as opposed to the reduced BDNF levels associated with increased age i.e adult counterparts. In view of reports demonstrating that

the adolescent brain is still undergoing widespread neural changes (Blakemore & Choudhury, 2006; Crews et al., 2007; Spear, 2000b) and that this age period is characterized by exploratory and learning behaviour necessary for survival of the young rat, the noted increase in our adolescent hippocampal BDNF levels is to be expected as this neurotrophin is essential for normal central nervous system development, neuronal plasticity and motivation and emotional responses (Lohof et al., 1993; Levine et al., 1995, 1998; Kossel et al., 2001). Moreover, a study by Swann and colleagues (1999) showed glutamatergic receptor-mediated morphological synaptic remodelling and pruning of excitatory axon collaterals and dendritic branches during the adolescent period.

The age-related decline in hippocampal BDNF in our study corresponds to clinical findings that demonstrated a reduction in BDNF concentration in late adulthood (Shirayama et al., 2002; Lommatzsch et al., 2004; Lapchak et al., 1993). Other human studies have also reported on this decline in circulating BDNF levels with an advance in age, suggesting a BDNF gene polymorphism is linked to the age-related hippocampal volume loss (Lommatzsch et al., 2004; Lapchak et al., 1993). Furthermore, animal studies have shown a decrease in adult DHC BDNF levels following exposure to trauma (Uys et al., 2006), suggesting a role for this neurotrophin in protecting the individual against trauma related behavioural hyperarousal. The present study confirmed that altered BDNF levels increase vulnerability to stressors by compromising hippocampal neurons which may lead to the development of behavioural abnormalities (McEwen, 1999).

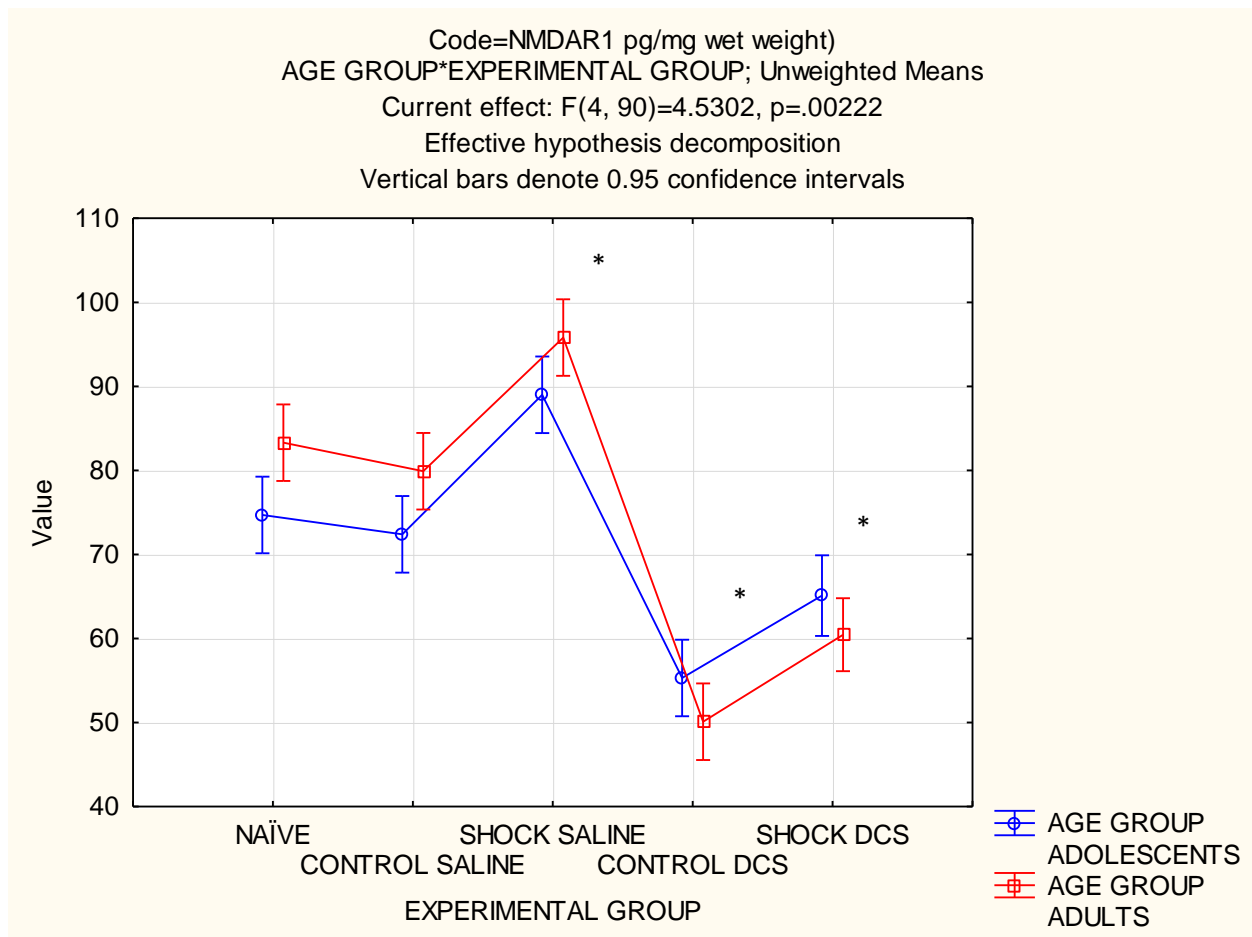


Figure 7.1: A line graph depicting NMDA-R1 protein concentrations in the dorsal hippocampus of naïve, control, shocked and DCS-treated animals. Values are expressed as unweighted means \pm SEM (n = 10) in pg/mg wet weight. *p \leq 0.05.

The NMDAR1 protein was expressed in a similar pattern for both age groups. Naïve and control rats (CS) had similar NMDAR1 levels. Shocked rats displayed significantly higher NMDAR1 concentrations (p<0.05/p=0.00, Bonferroni's Multiple comparison test). DCS treated shocked animals had significantly decreased NMDAR1 concentrations (p<0.05/p=0.00, Bonferroni's Multiple comparison test). Control DCS treated animals also had significantly lower NMDAR1 levels than the control saline-treated rats (p<0.05/p=0.00, Bonferroni's Multiple comparison test).

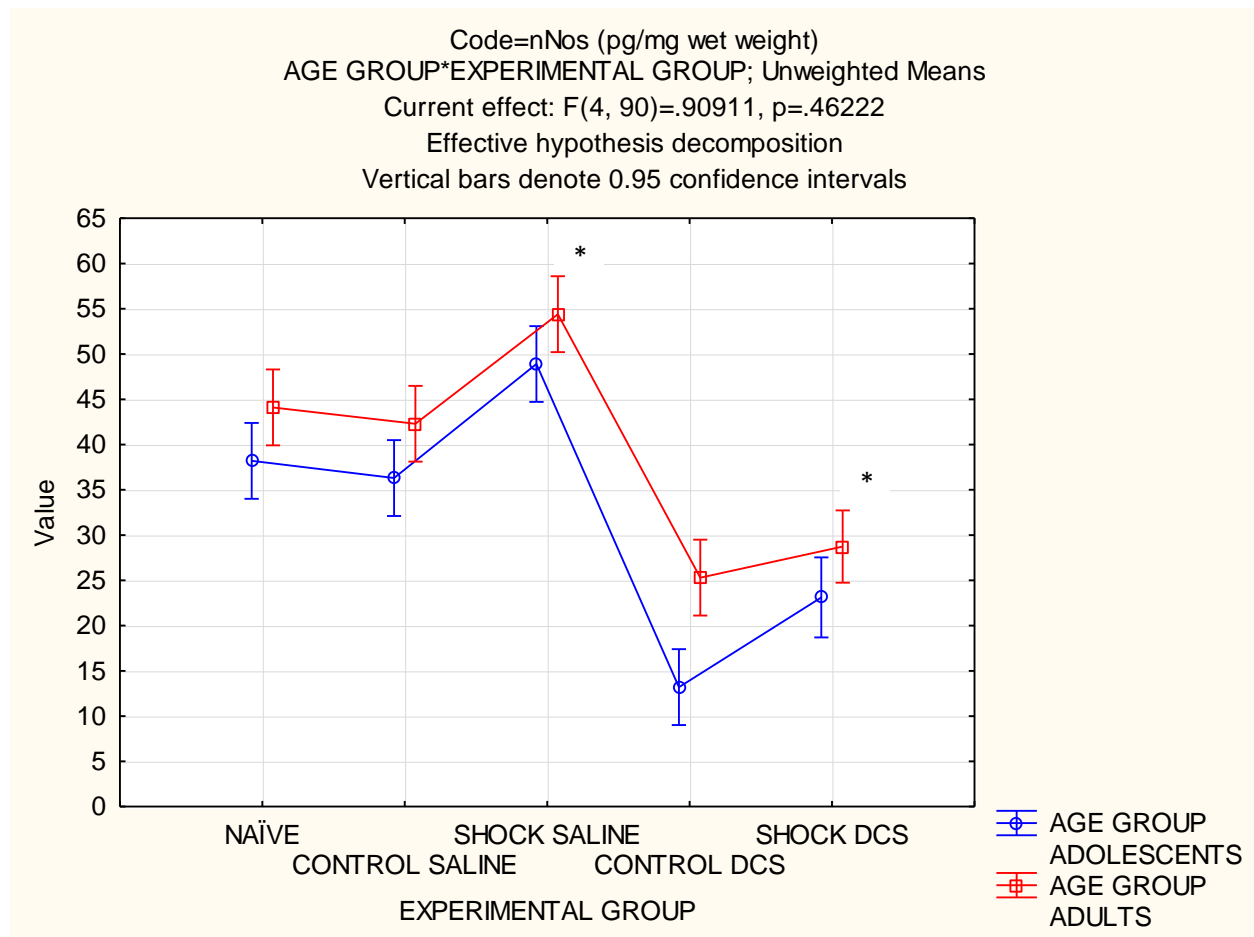


Figure 7.2: A line graph depicting nNOS protein concentrations in the dorsal hippocampus of naïve, control, shocked and DCS-treated animals. Values are expressed as unweighted means \pm SEM (n = 10) in pg/mg wet weight. *p \leq 0.05.

The nNOS protein was expressed in a similar pattern for both age groups. Naïve and control rats (CS) had similar nNOS levels. Shocked rats displayed significantly higher nNOS concentrations (p<0.05/p=0.00, Bonferroni's Multiple comparison test). DCS treated shocked animals had significantly decreased nNOS concentrations (p<0.05/p=0.00, Bonferroni's Multiple comparison test). Control DCS treated animals also expressed lower nNOS levels than the control saline-treated rats (p<0.05/p=0.00, Bonferroni's Multiple comparison test).

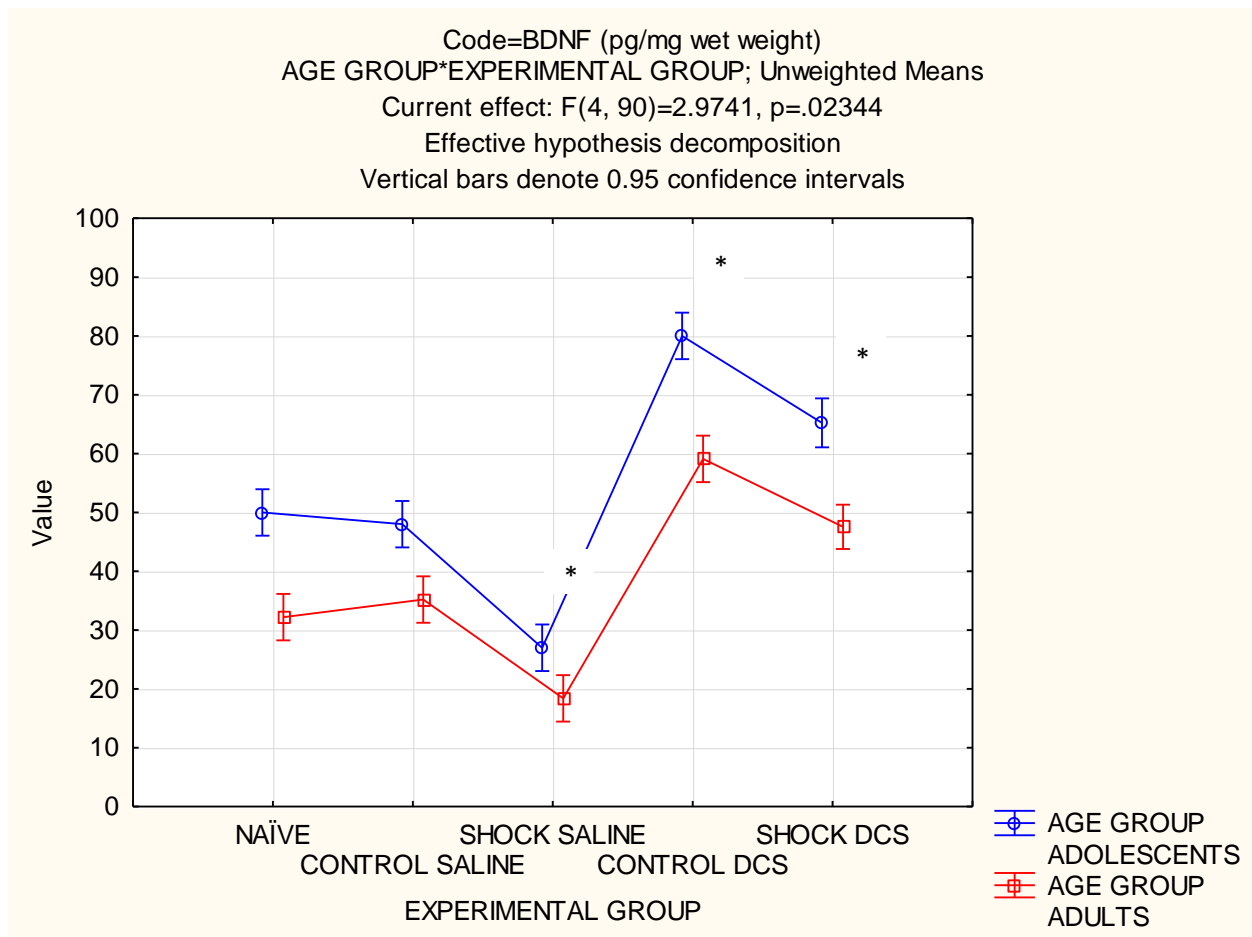


Figure 7.3: A line graph depicting BDNF protein concentrations in the dorsal hippocampus of naïve, control, shocked and DCS treated adolescent and adult animals. Values are expressed as unweighted means \pm SEM (n = 10) in pg/mg wet weight. *p \leq 0.05.

Adolescent and adult fear conditioned animals displayed significant decreases in BDNF concentrations compared to their controls (p = 0.02, Bonferroni's Multiple comparison test). DCS treated animals had significantly higher BDNF concentrations than saline-treated controls for both shocked and non-shocked groups (p = 0.02, Bonferroni's Multiple comparison test). Adults expressed significantly lower BDNF levels compared to adolescents (p = 0.02, Bonferroni's Multiple comparison test).

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Chapter 8

Proteomic profiling of the dorsal hippocampus of fear conditioned and DCS-treated rats

8.1 Introduction

In contextual fear conditioning a single training leads to LTM of context-aversive electrical foot shock association. It has also been proposed that fear extinction entails the process of learning. On a cellular level, a complex set of molecular changes (Hölscher 1999; Bliss & Collingridge 1993), involving LTP, was demonstrated to underlie hippocampal associative learning (Martin et al., 2000) and storage of memories in the HC. Moreover, animal studies have shown that long-term, but not short-term, memory requires the induction of new mRNA and protein synthesis (Dudai & Eisenberg, 2004). A growing body of evidence further demonstrated that common agents used in anxiety treatments alter mRNA expression of various signalling pathways (Lesch et al., 1992). We therefore decided to assess the molecular changes associated with our fear conditioning model and DCS treatment regime as well as identify the pathways upstream of protein synthesis which may be implicated in our animal model.

Proteomics involves the large scale studying of an entire protein population to determine a comprehensive view of the protein networks and their cellular function, post-translational modifications (Cantin & Yates, 2004) and interaction with signal transduction pathways (Grant & Blackstock, 2001) in pathology (Blackstock & Weir, 1999; Conrads et al., 2000; Choudhary & Grant, 2004). To determine the dorsal hippocampal proteins differentially expressed in both fear conditioned and DCS-treated rats, a proteomic technique involving isotope tags for relative and absolute quantification (iTRAQ) was employed. This method of isobaric tagging of peptides, iTRAQ, is a commonly used platform in mass spectrometry based quantitative proteomics, allowing determination of the amount of proteins from different sources in a single experiment (Ross et al., 2004; Gafken & Lampe, 2006; Zieske, 2006). iTRAQ allowed us to study up to four different conditions within a single experiment, making it very suitable to investigate pharmacological treatments in anxiety disorders (Ross et al., 2004).

8.2 Methodology

To determine the effects of trauma/fear conditioning and DCS treatment on dorsal hippocampal protein expression, we used 3 dorsal hippocampi from each experimental group (see Section 4.5.4 of Chapter 4 for more detail) in an 8-plex iTRAQ array. iTRAQ is a quantification method for labelling proteins in solution (see Section 4.5.4.2 of Chapter 4 for more detail). In brief the iTRAQ method is based on the covalent labelling of the N-terminus and side chain amines of peptides from protein digestions with tags of varying mass. In the current study the samples were fractionated by nano liquid chromatography and analyzed by tandem mass spectrometry. A database search was performed using fragmentation data to identify the labelled peptides and hence the corresponding proteins. The low molecular mass report ion was used to relatively quantify the peptides and proteins from which they originated.

8.2.1 Statistical analysis

For bioinformatics, acquired data was first processed using AB Sciex Protein Pilot (PP) version 4.0.8085 (Shilov et al., 2007). A peptide summary and a protein summary were produced using each of the eight iTRAQ labels as a denominator. In a parallel analysis, the raw output from the MS/MS was exported to the open mzML format using the AB Sciex data exporter version 1.1 beta. The mzML files were then converted to mzXML files using the msconvert utility from the ProteoWizard suite, release 2.2.3155. The mzXML files were run through the TransProteomic Pipeline (TPP) version 4.5r2 (Deutsch et al., 2010) using the X!Tandem analysis work flow. The output of the work flow was then processed to assign p-values to each of the calculated ratios. The significantly regulated proteins as determined by both TPP and PP were combined into summary data sheets. These files are quantification summaries containing ratios of each protein for each label over the denominator label. Two tailed z-tests were used, thus the p-values is of significance if below 0.025, not 0.05 as is common.

8.3 Proteomic results

The proteomic technique, an 8-plex iTRAQ, identified a total of 329 differentially expressed proteins in the adolescent (PND 33) and 240 differentially expressed proteins in the adult (PND 60) DHC of the various experimental groups.

8.3.1 Proteins common to naïve, PND33 controls and PND60 control rats

A comparison between the identified proteins for naïve, PND33 and PND60 rats, yielded 109 common peptides (Table 8.1, Figure 8.1). In general these proteins included functions spanning neuroplasticity, learning and memory, neuroprotection, Ca²⁺ homeostasis, signalling, oxidative stress, apoptosis, cellular transport, cellular structure and energy metabolism, and formed the core proteins of interest in subsequent comparisons.

8.3.2 Protein changes associated with fear conditioning (Control saline vs Shock saline)

A total of 38 proteins were identified when the group of shocked treated animals at PND 33 was compared to their controls (Table 8.2, Figure 8.2). The expression of 21 proteins was significantly increased while that of 17 proteins was significantly decreased. When similar comparisons were done for PND 60 rats, we found a total of 32 proteins differentially expressed in the shocked treated animals compared to their controls (Table 8.2, Figure 8.2). The expression of 17 proteins was significantly increased while that of 15 proteins was significantly decreased. Proteins such as calmodulin (decreased), albumin and haemoglobin (both increased) reflected similar profiles in both age groups, while adenosine triphosphatase (ATPase) expression was increased at PND 33 but reduced at PND 60.

Other proteins of relevance to fear conditioning that were also significantly altered in the PND 33 shocked rats included increased expression of glia maturation factor beta, microtubule-associated protein RB and sorting nexin 1 and 3, and reduced expression levels of neurofascin, neurofilament and neurabin 2. These proteins cover functions of neuronal structure, glial cell activation, endosomal protein sorting and inter-and

intracellular signalling. In the PND 60 rats the expression of calcineurin, annexin and hippocalcin was increased, while that of myelin basic protein, neural cell adhesion, brain acid soluble protein and synaptosomal associated protein was decreased.

8.3.3 Protein changes associated with D-cycloserine treatment alone (Control saline vs D-cycloserine)

A total of 18 proteins were identified when the group of DCS treated animals at PND 33 was compared to their controls (Table 8.3, Figure 8.3). The expression of 6 proteins was significantly increased while that of 12 proteins was significantly decreased. At PND 60 a total of 38 proteins were identified as differentially expressed between DCS treated and saline treated controls, with 23 proteins showing elevated expression and 15 displaying reduced expression (Table 8.3, Figure 8.3). Microtubule associated protein was the only protein that showed a common increase in both age groups, while neurogranin was the protein that was commonly decreased. In contrast hexokinase and histone 2B were increased in PND 33 rats but decreased in PND 60 animals.

In addition to these, other proteins that may provide insights into the mechanism of action of DCS, have also been identified. Of note in PND 33 animals were decreases in the levels of neurofascin and T complex (a molecular chaperone important for ATP hydrolysis), and in PND 60 rats decreases in the levels of profilin, synaptotagmin and synaptic vesicle binding protein. Increases in neural cell adhesion, neurochondrin and dynein were also noted in PND 60 rats.

8.3.4 Protein changes associated with D-cycloserine-treated shocked animals (Control DCS vs Shocked DCS)

A total of 31 proteins were identified when the group of DCS shocked treated animals at PND 33 was compared to their DCS treated controls (Table 8.4, Figure 8.4). Of these 21 proteins were significantly higher expressed, while the expression of a further 15 proteins were significantly diminished. For PND 60 rats 37 proteins were differentially expressed, with 21 showing elevated expression levels and 16 proteins had a down regulation in their expression (Table 8.4, Figure 8.4). Interestingly only the increased expression of glutamine synthetase was common between the two age groups of animals.

A number of other proteins were also identified that may have a bearing on the action of DCS in shocked animals. These include Lin7-homolog and tenascin that were increased in PND 33 rats; myelin basic protein and tweety that were decreased in PND 33 rats; profilin, hippocalcin, microtubule associated protein and neurogranin that were increased in PND 60 rats; and synaptotagmin that was decreased in PND 60 rats.

8.3.5 Overall protein changes when shocked animals are compared to DCS treated animals

Multiple comparisons of long lists of proteins generate a huge amount of data that goes beyond the scope of this section of the study. As an alternative a more simple approach was adopted in identifying proteins in shocked animals that were altered by DCS treatment. In doing so, some meaningful results, although limited, were obtained (Table

8.5). For instance in PND 33 rats the expression of tweety was increased by fear conditioning and this increase was reversed by DCS treatment. Contrary the same animals displayed decreased tubulin and beta adducin levels when shocked, and the expression of these proteins were increased by DCS. In PND 60 rats other proteins exhibited this pattern of expression, with hexokinase, cytochrome c and hippocalcin showing increased expression in shocked animals that were reduced by DCS treatment; and T complex, neural cell adhesion and tubulin displaying decreased expression upon shock exposure with DCS treatment normalising its expression.

8.4 Discussion

Since the onset of neuroproteomic studies (Kim et al., 2004), many housekeeping proteins have been identified in stress and anxiety disorders (Tanna et al., 1989; Kromer et al., 2005). With the utilization of nano liquid chromatography and tandem mass spectrometry in combination with iTRAQ labelling, the current study identified a protein profile which corresponds to previous published reports on the human and rodent brain proteome (Langen et al., 1999; Krapfenbauer et al, 2003; Stevens et al, 2003; Yang et al, 2004). Specifically, the protein data here mirrored previous rat hippocampal proteome findings from stress and anxiety studies conducted in our own laboratory (Uys et al., 2006; Faure et al., 2009).

The main effects of the trauma in the current study included alterations in structure and signalling proteins, cell cycle regulators and energy metabolism. Specifically, a decrease in the expression of energy metabolism, structural, neurogenesis and neuroprotective proteins was noted following the trauma. Along with these, an increase in oxidative stress

and apoptotic proteins were noted in shocked animals. Given that a contextual fear conditioning model was utilized, which involves associative learning and storing of fear memories in the HC, it is not surprising that learning and memory proteins were also differentially expressed in the shocked animals.

Of the 109 proteins found to be commonly expressed in both age groups of the current study, only a few proteins were commonly expressed in both the adolescent and adult animals in response to the trauma. These include a subunit of hexokinase-1, neurofilament polypeptide, T-complex, 2',3'-cyclic-nucleotide 3'-phosphodiesterase, serum albumin, calcium/calmodulin kinase II, prohibitin, heterogeneous ribonuclear protein, creatine kinase and a subunit of haemoglobin. As the abovementioned proteins form part of the previously identified common core proteins, the discussion will mostly focus on their role in relation to trauma and DCS treatment. It should be noted that other non-common proteins also differentially expressed in our study will be briefly mentioned with regards to processes underlying the trauma. These processes include fear learning and memory, NMDAR hypersensitization, neurotoxicity and apoptosis.

Haemoglobin for example, was one of the proteins that were increased by shock in both PND 33 and PND 60 rats. Haemoglobin is an oxygen ion transporter that has a high affinity for nitric oxide (NO) (Bellelli et al., 2006). All haeme-containing globin monomers bind NO metabolic products and thus we propose that the decreased levels of haemoglobin expressed in our fear conditioned rats contributed to the already present oxidative state created by increased activation of intracellular Ca^{2+} cascades. This explanation would be in line with previous studies indicating a protective role of haemoglobin against nitrosative stress (Wilson & Reeder, 2008).

The expression levels of albumin were similarly increased as haemoglobin. Albumin is the most abundant protein in the body, and generally reflects the nutritional status of an individual. Albumin is synthesized by microglial cells in the brain which is enhanced when neurons are under threat. Ahn et al. (2008) demonstrated increased albumin production by microglia exposed to lipopolysaccharide or beta-amyloid toxicity. It is therefore plausible that in our animals, albumin synthesis was increased to combat the deleterious effects of foot shock during fear conditioning. Alterations in ATPase activity in shocked animals suggest that the hippocampi of shocked animals were under energy stress.

Clinical research demonstrated that PTSD patients presented with enhanced hippocampal activation during associative memory and learning (Geuze et al., 2008), with earlier studies implicating a NMDAR-dependent mediation of many forms of active learning (Morris, 1989; Flood et al., 1990; Fanselow & LeDoux, 1999). Indeed our neurochemical and behavioural data (See Chapters 5 and 7) confirm that associative learning occurred as evidenced by the overstimulation of hippocampal NMDARs and an increased fear response of the traumatized rats. Moreover, upon re-exposure to the context, fear conditioned rats displayed increased freezing which is indicative of consolidation of the initial fear memory i.e. LTM. Molecular studies have previously demonstrated that acquisition or consolidation of LTM requires activation of protein kinases such as PKA and MAPK, which activate transcription factors such as CREB and ERK-1 for new mRNA-, protein synthesis and synapse formation (Schafe & LeDoux, 2000). However the current data suggests that other proteins may be equally important in the development of fear conditioning in rats.

Animals subjected to foot shock displayed increased expression of glia maturation factor beta, microtubule-associated protein RB and sorting nexin 1 and 3 for PND 33 rats; and calcineurin, annexin and hippocalcin for PND 60 rats, while reduced expression levels of

neurofascin, neurofilament and neurabin 2 (PND 33) and myelin basic protein, neural cell adhesion, brain acid soluble protein and synaptosomal associated protein (PND 60) were obtained. These proteins that cover functions of neuronal structure, glial cell activation, endosomal protein sorting and inter-and intracellular signaling, reflect the various molecular entities that are affected by foot shock and underpin the complexity of fear conditioning. For instance, increased expression of glia maturation factor enhances p38 MAP kinase activity that can lead to the increased activation of transcription factors such as CREB with downstream initiation and production of nerve growth factors including BDNF (Lim & Zaheer, 2006). Previous studies have implicated the MAP kinase signaling pathway in associative learning and the formation of contextual fear memories (Atkins et al., 1998; English & Sweatt, 1996).

Similarly a link between proteins such as calcineurin (a serine/threonine phosphatase), annexin (a calcium-binding protein) and hippocalcin (a calcium sensor in pyramidal cells of the hippocampus) that are involved in calcium/calmodulin-dependent signalling, and CREB-mediated learning and memory processes, can be easily envisioned. Surprisingly significant decreases were observed in the expression of proteins whose functions relate to neuronal structure e.g. neurofilament – a cytoskeletal protein (Yuan et al., 2012) and neurabin 2 - an actin-binding protein (Satoh, 1998), as one would have expected a high degree of neural plasticity in fear conditioned rats.

The multi-prong stimulation of protein biosynthesis via CREB signalling, reflects its importance in memory consolidation. Pioneering work of Grecksh and Matthies in 1980 showed that memory consolidation relies on two critical protein synthesis-dependent

macromolecular phases. Since then various other researchers have demonstrated that coactivation of these protein synthesis periods are dynamic as they are dependent on the level of contextual fear training. Further corroborating the role for the formation of new proteins in memory synaptic process, are studies demonstrating that synthesis of new hippocampal proteins underlie fear-motivated memory formation (Scholey et al., 1993; Freeman et al., 1995; Rose, 2000; Igaz et al., 2002). Specifically during the second consolidation phase, new proteins were shown to be synthesized and glycosylated; leading to the establishment of an enduring memory trace i.e. LTM.

Interestingly, only long- and not short-term memory formation requires new mRNA and proteins to be synthesized (Dudai & Eisenberg, 2004). Subsequent behavioural and genetic studies emphasized the dependence of long-term contextual fear memory formation on hippocampal mRNA and protein synthesis (Mizuno & Giese, 2005).

The present proteomic findings suggest that contextual fear conditioning may lead to large scale hippocampal cellular distress. As discussed in the literature review, in addition to inducing LTP, trauma-induced NMDAR hyperexcitability may also lead to excitotoxicity. A link between psychosocial and physical stress and mitochondrial dysfunction has been previously documented. We therefore propose that dysregulated Ca^{2+} metabolism mediates mitochondrial oxidative damage by activating downstream phospholipases, endonucleases and proteases as well as increasing free radical production by excessively activating nNOS.

Compared to the controls, fear conditioned animals showed a decrease in calcium homeostatic proteins, dysregulated signalling pathways with ensuing alterations in cytoskeletal proteins, decreased neuroplasticity regulators and increased apoptotic initiator proteins. These proteins include, but were not limited to hippocalcin, neuromodulin, microtubule associated protein, synaptotagmin, peroxiredoxin and prohibitin. The proteomic data supported our neurochemical findings in that a simultaneous increase in NMDARs and apoptotic proteins was observed following the trauma. Given that Ca^{2+} metabolism is related to oxidative damage, dysregulation of Ca^{2+} flux produces more oxidizing free radicals and nitrosative species that are extremely damaging to cells. Proteins other than those involved in Ca^{2+} homeostasis were also found to be differentially expressed in fear conditioned rats. For instance cytochrome b-c complex, a pro-apoptotic protein that regulates the organisms innate defence system (Leto & Geiszt 2006), has a crucial function of producing reactive oxygen species (ROS) such as superoxide. It is therefore possible that proteins such as cytochrome b serves to heighten the already present oxidative stress state within the HC. Such a possibility is plausible since a few studies have implicated the cytochrome b-mediated oxidative stress in anxiety and mood disorders (Hovatta et al., 2005; Rammal et al., 2008; Masood et al., 2008; Schiavone et al., 2009; Nair et al., 2011), while others have confirmed the cytochrome b-ROS involvement in psychiatric disorders such as anxiety disorders (Gard, 2004; Tsaluchidu et al., 2008; Sorce and Krause, 2009; Andrezza et al., 2009; Steckert et al., 2010).

Analysis of the current data also revealed significant effects of fear conditioning with respect to energy metabolism. A number of metabolic enzymes were found to be dysregulated following the trauma (i.e. footshock). These included ATPase, pyruvate dehydrogenase, hexokinase and creatine kinase, which may reflect a general dysfunction of the cell and its inability to supply adequate energy to function optimally. Deficits in

energy metabolism may consequently have a severe impact on voltage-dependent neurotransmission, resulting in abnormal neuronal functioning. Mitochondria are known to be the centers of energy production and a recent paper by Streck et al (2013) have described the role of defective mitochondria in the development of psychiatric disorders.

Our behavioural data suggests that DCS treatment in conjunction with fear extinction was able to normalise the anxiolytic effects of fear conditioning in rats. DCS is a partial NMDA receptor agonist and therefore it is possible that our behavioural observations resulted from the effect of DCS on the glutamatergic system in the brain. This would also bring our findings in line with reports showing the counteractive effects of DCS on NMDAR overstimulation (Naskar & Dreyer, 2001). Furthermore preclinical studies utilizing inhibitors of glutamate reuptake to induce increased glutamate levels in the synaptic cleft, ultimately documented a decrease in NMDARs postsynaptically (Cebers et al., 1999).

Promotion of NMDAR activity usually leads to activation of targets downstream of the receptor. Of these, BDNF has been well studied. In the present study BDNF levels of DCS treated rats were significantly increased. This observation was in line with reports showing activation of the NMDA receptor resulting in increased BDNF expression (Marini et al., 1998; Yaka et al., 2003). This finding further suggests that DCS might act via BDNF and its downstream signalling pathways in enhancing fear extinction. Considering the essential role of BDNF in synaptic plasticity (Lu et al., 2014), we hypothesize that the modulation of this neurotrophin by DCS during fear extinction might increase the growth and differentiation of DHC neurons and thus strengthen their connections resulting in greater enhancement of fear extinction. BDNF may perform this function at various molecular levels. BDNF has been shown to stimulate phosphorylation of neurofilament

(Tokuoka et al., 2000). Thereby rescuing neurofilament loss (Hayes et al., 1995) attributed to fear conditioning.

Our proteomic results suggest alternative ways by which DCS could have enhanced fear extinction. The expression of proteins such as tweety, hexokinase, T-complex, cytochrome c and hippocalcin was all elevated by fear conditioning and this increase in expression was not seen in DCS treated animals. Similarly fear conditioning appeared to have reduced the expression of neural cell adhesion, tubulin and beta-adducin, while DCS was able to reverse this decrease. These proteins represent functions that include Ca^{2+} homeostasis and/or Ca^{2+} -dependent neurotransmission, energy metabolism, cell signalling and neuronal structure. It therefore appears as if DCS may act on several molecular pathways and that collectively these effects may facilitate restoration of behavioural function.

Tweety proteins are chloride channels that are important to maintain membrane potentials. Aberrant tweety function has been associated with epileptic activity (He et al., 2008). T-complex is a molecular chaperone and is involved in ATP hydrolysis. Together with increased levels of hexokinase, elevated T-complex concentrations reflect the need of fear conditioned cells for energy. Tubulin and beta-adducin on the other hand, are proteins that are important for normal cytoskeletal functioning and alterations in their expression further highlights the severe stress that the brain experiences during fear conditioning.

8.4.1 *Neurabin-2: adolescent protein*

Studies have shown that the brain continues to develop throughout the adolescent period (Markus & Petit, 1987; Sowell et al., 1999) and that the enhanced vulnerability to trauma during adolescence is due to glutamatergic neurotransmission, in particular the NMDA receptor system.

Adolescent rats in the present study, displayed slightly higher BDNF levels as opposed to their adult counterparts. In view of reports demonstrating that the adolescent brain is still undergoing widespread neural changes (Blakemore & Choudhury, 2006; Crews et al., 2007; Spear, 2000) and that this age period is characterized by exploratory and learning behaviour necessary for survival of the young rat, the noted increase in our adolescent hippocampal BDNF levels is to be expected as this neurotrophin is essential for normal central nervous system development, neuronal plasticity and motivation and emotional responses (Lohof et al., 1993; Levine et al., 1995, 1998; Kossel et al., 2001). Moreover, a study by Swann and colleagues (1999) showed glutamatergic receptor-mediated morphological synaptic remodelling and pruning of excitatory axon collaterals and dendritic branches during the adolescent period.

Interestingly, the protein neurabin-2 was only expressed in adolescent rats. Specifically, a significant increase was observed in neurabin-2 protein expression levels following the fear conditioning protocol of the present study. Neurabin-2, also known as spinophilin, is an actin filament binding protein implicated in excitatory synaptic transmission. Given that neurabin-2 activity is known to be modulated by Ca^{2+} , we propose that the overactivation of NMDARs (i.e. increased NMDAR1 levels) observed in our fear conditioned adolescent rats led to increased expression of this synaptic actin-binding protein. Researchers have determined targets of neurabin-2 such as the downstream component of the rapamycin

pathway, p70S6 kinase and protein phosphatase-1 (PP-1) (Baudry et al., 2000). Furthermore, a recent study by Hou and colleagues demonstrated that synaptic NMDAR stimulation activated PP-1 (Hou et al., 2013). Similar to Hou et al. (2013), we also found an increased expression of PP-1 upon overstimulation of NMDARs in the fear conditioned rats of our study. In addition, the adolescent shocked rats presented with increased expression of calcium/calmodulin-dependent protein kinase type II (CaMKII) levels. We therefore postulate that during fear conditioning, NMDARs are hypersensitized causing increased intracellular concentration of calcium. The calcium induces CaMKII which in turn phosphorylates neurabin-2 and detaches it from the actin filaments. The neurabin-2 protein is therefore able to target PP-1 within the post synaptic cell. As previously mentioned, the synaptic process of LTP is induced by hippocampal NMDAR activation. Taken together, this data implicates the neurabin-PP1 complex, via NMDAR activation and consequent calcium-calmodulin binding, in hippocampal glutamatergic synaptic plasticity. The 2006 study of Xiao and colleagues supports the idea of an actin-binding-serine/threonine phosphatase complex in mediating hippocampal synaptic plasticity (Xiao et al., 2006). This molecular finding confirms the role of neurabin-2 in anxiety i.e. increased neurabin-2 levels result in anxiety-like symptoms. Moreover, we infer that neurabin-2 plays a role in associative learning as we observed increased freezing responses in traumatized rats during the fear conditioning and associative test exposures. We conclude that further research is needed on this synaptic scaffolding protein to shed light into the events dictating brain development during trauma exposure.

8.5 Conclusion

Our proteomics data suggests that fear conditioning caused NMDAR overstimulation, resulting in excessive increases of intracellular Ca^{2+} concentrations and the dysregulation

of many signaling pathways. Subsequently the cell structure is disrupted and cell death mechanisms are initiated. These pathophysiological processes culminate in abnormal behaviour as displayed by the shocked rats, thereby confirming aberrant brain function. The administration of DCS during the fear extinction period was able to reverse the abnormal behaviour and it did so by restoring the expression levels of a number of proteins, besides that of NMDARs.

The molecular effects of fear conditioning were different in the two age groups of rats with very few common proteins being identified. A similar protein expression pattern was observed when DCS-treated rats were compared across age. These observations strongly suggested that the normal neurophysiology of adolescents is significantly different to that of adults and that the age factor must be considered when patients present with stress and anxiety-related disorders.

Table 8.1: A list of proteins identified to be common in both age groups (PND 33 & PND 60) with respect to Naive groups, together with their most common functions

Protein	Function
Guanine deaminase	Neuroplasticity
Calcium/calmodulin-dependent protein kinase type II alpha chain	Neuroplasticity, NMDAR signaling, LTP
Neurogranin	Neuroplasticity, Learning, Ca ²⁺ Homeostasis
Glutamate dehydrogenase 1, mitochondrial	Learning and Memory
Gamma-enolase	Neuroprotection
Vesicle-associated membrane protein 2	Ca ²⁺ Homeostasis
Synaptotagmin-1	Ca ²⁺ Homeostasis
Isoform 2 of Calcineurin subunit B type 1	Ca ²⁺ Homeostasis
Calmodulin	Ca ²⁺ Homeostasis
Neuron-specific calcium-binding protein hippocalcin	Ca ²⁺ Homeostasis/Signaling
Dual specificity mitogen-activated protein kinase kinase 1	Ca ²⁺ Homeostasis/Signaling
Guanine nucleotide-binding protein Alpha-2	Signaling
LIM and SH3 domain protein 1	Signaling
14-3-3 protein epsilon, delta, gamma & zeta	Signaling, Neuroplasticity
60 kDa heat shock protein, mitochondrial	Signaling (Chaperone)
Calreticulin	Signaling (Chaperone), Ca ²⁺ -binding
Heat shock cognate 71 kDa protein	Signaling (Chaperone)
Stress-70 protein, mitochondrial	Signaling (Chaperone), Cell proliferation
10 kDa heat shock protein, mitochondrial	Signaling (Chaperone), Stress response
Heat shock protein HSP 90- alpha & beta	Signaling (Chaperone), Stress response
Heat shock protein 105 kDa	Stress response
Peptidyl-prolyl cis-trans isomerase A	Oxidative stress, Protein-binding
Superoxide dismutase [Mn], mitochondrial	Cellular stress (Antioxidant)
Peroxiredoxin-2	Cellular stress (redox regulation, antioxidant), Apoptosis
Hexokinase-1	Apoptosis
Fructose-bisphosphate aldolase C	Apoptosis, Aging, Metabolism

Table 8.1: A list of proteins identified to be common in both age groups (PND 33 & PND 60) with respect to Naive groups, together with their most common functions

Protein	Function
Voltage-dependent anion-selective channel protein 1	Apoptosis, Transport
Acyl-CoA-binding protein	Transport
Alpha-synuclein	Transport
Serum albumin	Transport
Mu-crystallin homolog	Transport
V-type proton ATPase subunit E 1	Transport
4F2 cell-surface antigen heavy chain	Transport
Isoform II of V-type proton ATPase 116 kDa	Transport (Hydrogen ions)
Apolipoprotein E	Transport (Lipid)
Vesicle-fusing ATPase	Transport (vesicle-mediated), Neurotransmitter secretion
Isoform Non-brain of Clathrin light chain B	Transport (Neurotransmitter)
Hemoglobin subunit alpha-1, 2 & beta-1	Transport (Oxygen)
Syntaxin-1B	Transport, Vesicular docking and fusion
Ras-related protein Rab-3A	Transport (Neurotransmitter), Axonogenesis
2',3'-cyclic-nucleotide 3'-phosphodiesterase	Axonogenesis, Metabolism (RNA), Nuclear, Aging
Prohibitin	Nuclear, Stress response, Aging
Glyceraldehyde-3-phosphate dehydrogenase	Nuclear, Apoptosis
Heterogeneous nuclear ribonucleoprotein A1	Nuclear (RNA binding)
Isoform A2 of Heterogeneous nuclear ribonucleoproteins A2/B1	Nuclear (RNA binding)
Histone H3.3	Nuclear (DNA binding), Brain development
Non-erythroid spectrin beta	Structure (Actin-binding)
Tubulin alpha-1A, 4A & 1B chain	Structure
Tubulin beta-2A, 5A & 2C chain	Structure
Microtubule-associated protein 1A	Structure

Table 8.1: A list of proteins identified to be common in both age groups (PND 33 & PND 60) with respect to Naive groups, together with their most common functions

Protein	Function
Isoform Adducin 63 of Beta-adducin	Structure, Calmodulin binding
Alpha-internexin	Structure, Neurogenesis
Neuromodulin	Neurogenesis
Isoform E1 of Drebrin	Neurogenesis (Immature rat brain)
Dihydropyrimidinase-related protein 1, 2 & 4	Neurogenesis, Cytoskeleton
Protein bassoon	Cytoskeleton organization
Protein kinase C and casein kinase substrate in neurons protein 1	Dendrite development, Endocytosis
Endophilin-A1	Dendrite development (BDNF-induced), Endocytosis
Elongation factor Tu, mitochondrial	Protein biosynthesis
Transitional endoplasmic reticulum ATPase	Nuclear
ADP-ribosylation factor 3	Nuclear, Transport
Brain acid soluble protein 1	Nuclear, Calmodulin-binding
ATPase inhibitor, mitochondrial	Calmodulin-binding
Malate dehydrogenase, cytoplasmic	Metabolism (Energy)
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	Metabolism (Energy)
Glycogen phosphorylase, brain form	Metabolism (Energy)
Phosphoglycerate kinase 1	Metabolism (Energy)
Triosephosphate isomerase	Metabolism (Energy)
Aconitate hydratase, mitochondrial	Metabolism (Energy)
ATP synthase subunit alpha, mitochondrial	Metabolism (Energy)
Sodium/potassium-transporting ATPase subunit alpha-1 & 3	Metabolism (Energy)
Sodium/potassium-transporting ATPase subunit beta-1	Metabolism (Energy)
Dihydrolipoyl dehydrogenase, mitochondrial	Metabolism (Energy)
Alpha-enolase	Metabolism (Energy)
Creatine kinase B-type	Metabolism (Energy)
Pyruvate kinase isozymes M1/M2	Metabolism (Energy)
Phosphatidylethanolamine-binding protein 1	Metabolism (Energy)

Table 8.1: A list of proteins identified to be common in both age groups (PND 33 & PND 60) with respect to Naive groups, together with their most common functions

Protein	Function
Creatine kinase, mitochondrial 1, ubiquitous	Metabolism (Energy)
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Metabolism (Energy)
L-lactate dehydrogenase B chain	Metabolism (Energy)
Glucose-6-phosphate isomerase	Metabolism (Energy)
Phosphoglycerate mutase 1	Metabolism (Energy)
Citrate synthase, mitochondrial	Metabolism (Energy)
Malate dehydrogenase, mitochondrial	Metabolism (Energy)
6-phosphofructokinase type C	Metabolism (Energy)
ATP synthase subunit delta, mitochondrial	Metabolism (Energy), Transport
V-type proton ATPase subunit B, brain isoform	Metabolism (Energy), Transport
ATP synthase subunit beta, mitochondrial	Metabolism (Energy), Ca ²⁺ ion binding
Fructose-bisphosphate aldolase C	Metabolism (Energy), Protein scaffolding
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Metabolism (protein degradation)
Nucleoside diphosphate kinase B	Metabolism (Nucleotide), Oxidative stress response
Synapsin-2	Metabolism Cytoskeleton, Neurotransmitter release
Adenylate kinase isoenzyme 1	Metabolism (Energy), Neuron differentiation
Histone H4	Cell differentiation
Aspartate aminotransferase, cytoplasmic	Metabolism (Glutamate, Aspartate)
Glutamine synthetase	Metabolism (Neurotransmitter), Glutamate binding
Spectrin alpha & beta chain, brain 2	Synaptic vesicle docking, cytoskeleton, Ca ²⁺ Homeostasis
Amphiphysin	Endocytosis (synaptic vesicle), Learning
Clathrin heavy chain 1	Endocytosis, Heat shock protein binding
Ubiquitin	Endocytosis, Signaling
Ubiquitin thioesterase	Immunity, DNA repair
Septin-7	Cell cycle
Dynactin subunit 2	Synapse formation (during development)
Cytoplasmic dynein 1 heavy chain 1	Structure (Motor protein)
Ubiquitin-like modifier-activating enzyme 1	Protein modification
Peptidyl-prolyl cis-trans isomerase FKBP1A	Protein folding, Ca ²⁺ channel activation

Table 8.2: A list of proteins identified to be differentially expressed in both age groups (PND 33 & PND 60) with respect to control+saline and shock+saline. Protein fold increase and decrease range from 0.5 to 2 and -0.5 to -2 respectively.

PND 33		PND 60	
Proteins Increased	Proteins Decreased	Proteins Increased	Proteins Decreased
lin-7 homolog	neurofascin	serum albumin	hexokinase
glia maturation factor	14-3-3 protein theta	ribonuclear protein	2',3' cyclic nucleotide
sorting nexin	cell division control	cytochrome c	myelin basic protein
serum albumin	ribosomal protein	glu-6 phosphate	neural cell adhesion
NADH	prot phosphatase 1A	t-complex	ATPase
hexokinase	tumor D54	dynamamin	Na/K transport ATPase
tweety	tubulin	electron transfer flavoprotein	calcium transport ATPase
haemoglobin	neurofilament	calcineurin	histone
proteasome	Apolipoprotein E	ras-related protein	voltage dependent anion
prothymosin	Neurabin 2	annexin	brain acid soluble protein
fatty acid binding protein	phoshopglucomutase	haemoglobin	synaptosomal assoc prot
microtubule associated protein	Prohibitin	peptidyl cis-trans	alpha internexin
tropomodulin	ser/threonine	glyceraldehyde	cytochrome c oxidase
actinin	adducin	transgelin	calmodulin
peroxiredoxin	Apo E	hippocalcin	disk large homolog
creatine kinase	calmodulin	Wiskott-Aldrich syndrome protein	
ATPase	2',3' cyclic nucleotide	guanine nucleotide binding protein	
ribonuclear protein			
succinate dehydrogenase			
aspartate aminotransferase			
parathymosin			

Table 8.3: A list of proteins identified to be differentially expressed in both age groups (PND 33 & PND 60) with respect to control+saline and control+DCS. Protein fold increase and decrease range from 0.5 to 2 and -0.5 to -2 respectively.

PND 33		PND 60	
Proteins Increased	Proteins Decreased	Proteins Increased	Proteins Decreased
ribonuclear prot microtubule assoc prot proteasome hexokinase L-lactate dehydrogenase histone	T-complex tweety neurogranin neurofascin SNAP25 NADH cell division tumor d54 UDP cell division ctrl d-dopachrome complement C	brain acid soluble prot neural cell adhesion creatine kinase amphiphysin coactosin haemoglobin myelin basic prot Wiskott-Aldrich histone mu-crystallin tubulin non-thyroid spectrin NAD ser/threonine spna microtubule assoc prot glu-6 phosphate neurochondrin spectrin beta calcium transport ATPase dynein prot disulfide voltage dependent anion	cytochrome c Na/K transport ATPase ATPase profilin guanine nucleotide binding protein hippocalcin histone synaptic vesicle binding prot neurogranin inner membrane prot zero beta globin hexokinase synaptotagmin beta adducin amine oxidase

Table 8.4: A list of proteins identified to be differentially expressed in both age groups (PND 33 & PND 60) with respect to control+DCS and shock+DCS. Protein fold increase and decrease range from 0.5 to 2 and -0.5 to -2 respectively.

PND 33		PND 60	
Proteins Increased	Proteins Decreased	Proteins Increased	Proteins Decreased
fam49b prot	GrpE	profilin	neural cell adhesion
Pkc2 prot	phosphoglucomutase	hippocalcin	mu-crystallin
peroxiredoxin	ATP synthase	glutathione S transferase	ATPase
NADH	prot kinase C	tubulin alpha	hexokinase
tenascin	myelin basic prot	ser/threo	complexin
Lin-7 homolog	tweety	creatine	ribonuclear prot
glutamine synthetase	cell division ctrl	glu-6	histone
hexokinase	eukariotic initiation factor	haemoglobin	ubiquin
complement c	T-complex	coactisin	Na/K ATPase transport
40-ribosomal prot	phosphacan	phosphoglycerate	amine oxidase
prot IMPACT	WFS1	myelin	histone
HSP co chaperon 90		beta adducin	synaptotagmin
glycoprot m6a		microtubule assoc prot	inner membrane prot
tropomodulin		NAD	guanine nucleotide binding prot
myosin-Va		glutamine synthetase	cytochrome
proteasome		electron transfer flavoprot	voltage dependent anion
alanyl tRNA synthetase		dynamin	
HSP 10		2',3' cyclic	
fatty acid binding prot		neurogranin	
glutamate decarboxylase		Wiskott-Aldrich	
		non erythroid spectrin	

Table 8.5: A list of proteins identified to be differentially up- and downregulated in response to shock exposure and DCS treatment in both age groups (PND 33 & PND 60).

PND 33		
Protein	Shock	DCS
Tweety	↑	↓
Tubullin	↓	↑
Beta-adducin	↓	↑
PND 60		
Protein	Shock	DCS
T-complex	↑	↓
Hexokinase	↑	↓
Cytochrome c	↑	↓
Hippocalcin	↑	↓
Neural cell adhesion	↓	↑

Figure 8.1: Venn diagram illustrating the number of distinct and common (overlapping region of the diagram, n=109) DHC proteins identified in both age groups (PND 33; purple, PND 60; turquoise) with respect to Naïve groups.

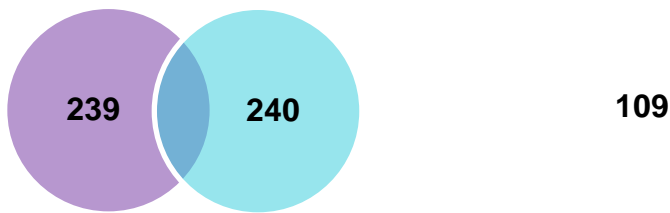


Figure 8.2: Venn diagram illustrating the number of distinct and common (overlapping region of the diagram, n=7) DHC proteins identified in both age groups (PND 33; blue, PND 60; yellow) with respect to control+saline and shock+saline groups.

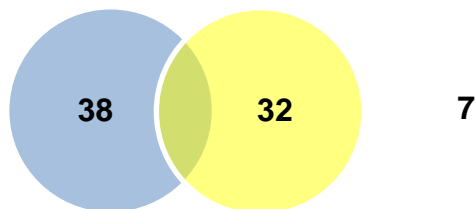


Figure 8.3: Venn diagram illustrating the number of distinct and common (overlapping region of the diagram, n=3) DHC proteins identified in both age groups (PND 33; blue, PND 60; pink) with respect to control+saline and control+DCS groups.

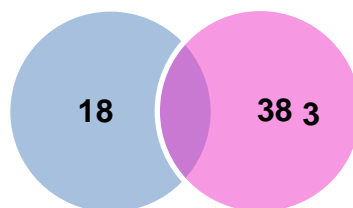
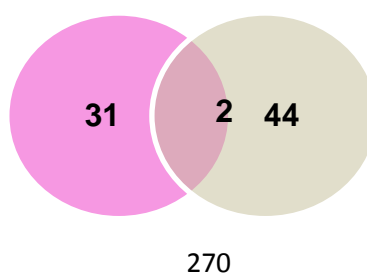


Figure 8.4: Venn diagram illustrating the number of distinct and common (overlapping region of the diagram, n=2) DHC proteins identified in both age groups (PND 33; pink, PND 60; grey) with respect to control+DCS and shock+DCS groups.



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Chapter 9

General Conclusion

The management of PTSD remains problematic, partly due to gaps in our current understanding of its aetiology and partly due to the lack of effective treatment options. The subsequent aim of the present study was to add to the existing body of knowledge about the pathophysiology of PTSD and to elaborate on the scientific basis for the use of DCS as an alternative treatment strategy for PTSD. The focal point of the study was therefore to investigate the effects of trauma on adolescents and adults and whether DCS can rescue these effects. An animal model of PTSD was established and examined on a behavioural, neurochemical and proteome level.

The fear conditioning model of the current study can be considered a valid animal model for use in researching clinical PTSD. As evidence, animals exposed to foot shock displayed a significant fear conditioned response when tested for associative and non-associative memories. These two memory processes have been demonstrated to underlie PTSD symptomatology. Additionally, fear conditioned rats presented with various anxiety-like symptoms such as anxiousness, hyperarousal and anhedonia as measured by different behavioural tests (e.g. open field, light/dark box and forced swim test).

Based on preliminary results on the therapeutic efficacy of DCS treatment for anxiety disorders in humans, we hypothesized that DCS was able to rescue the footshock elicited PTSD-like symptoms exhibited in our fear conditioned rats. We demonstrated that DCS was effective in diminishing the fear conditioned response (e.g. less freezing, less anxiety-like and hyperarousal symptoms) in shocked animals. This positive effect of DCS on anxiety disorders confirmed earlier findings of other researchers utilizing different stress methodologies.

Molecular studies have proposed that increased oxidative stress (Diehl et al., 2012) and subsequent apoptosis (Czeh & Lucassen, 2007), as well as a reduction in hippocampal neurogenesis and gliogenesis (DeCarolis & Eisch, 2010; McEwen, 1999), contributes to trauma-induced hippocampal volume loss. In addition, hippocampal alterations have been shown to manifest as aberrant behaviour. In line with the above, the current neurochemical measurements revealed the deleterious effects of elevated Ca^{2+} concentrations and excessive nNOS production upon NMDAR overactivation. A consequent increase in oxidative proteins and decrease in structural proteins was also noted in the fear conditioned rats. Therefore our findings confirm a role for these proteins in hippocampal shrinkage and subsequent trauma related behaviour, as observed in the shocked animals.

Additionally, we reported a down regulation in BDNF levels of the fear conditioned animals. Various animal stress studies, including footshock studies (Rasmusson et al., 2002; Duman, 2002), have demonstrated decreased hippocampal BDNF activity (Nibuya et al., 1999; Rasmusson et al., 2002; Tsankova et al., 2006; Lippmann et al., 2007; Nair et al., 2007; Bazak et al., 2009). Based on our decreased BDNF finding and the literature which implicates this neurotrophic factor in neuron differentiation and survival, we propose that the hippocampal integrity is compromised by impaired neurogenesis and this abnormality manifests in fear related memories. In support, we observed an associative fear memory retention and simultaneous decrease in neuroplastic proteins in our fear conditioned rats (Benraiss et al., 2001; Lee et al., 2001; Pencea et al., 2001; Katoh-Semba et al., 2002). Corroborating this notion are behavioural paradigms which observed a concurrent decrease in BDNF signalling and impaired LTP (Patterson et al., 1996; Monteggia et al., 2004) and learning and memory (Lu et al., 2008).

The proteomics data suggests that fear conditioning caused NMDAR overstimulation leading to excessive intracellular calcium concentrations with subsequent dysregulation of various signaling pathways. It is therefore possible that impaired intracellular signaling may underlie the PTSD-like symptoms exhibited by the shocked rats. We also observed trauma-induced decreases in structural and synaptic proteins of fear conditioned rats in the current study. In particular, neurofilament protein, microtubule associated protein 2 (MAP-2) and glial fibrillary acidic protein (GFAP) expression was decreased in response to the brief yet acute footshock. These proteins have respectively been identified as neuronal, dendritic and glial markers. Given their biomarker role and the observed changes in these structural proteins of the shocked rats, we therefore infer that alterations in the hippocampal grey matter (e.g. neuronal and glial cell loss) may contribute to the trauma-induced hippocampal shrinkage noted in humans and animals presenting with PTSD symptoms. Converging evidence supports the role of an impaired hippocampus in PTSD. Thus our observed alterations in differentially expressed hippocampal proteins confirm the role of aberrant hippocampal functioning in PTSD.

In contrast, DCS administration in conjunction with fear extinction was able to reverse the abnormal anxiety-like behaviour of the shocked rats, by restoring the expression levels of a number of structural proteins. Studies have also implicated an interaction between BDNF and structural proteins, specifically protein neurofilament (Tokuoka et al., 2000). Immunohistochemical studies investigating the effect of BDNF (exogenous) on injured neurons, demonstrated an increase in neurofilament protein phosphorylation (Hayes et al., 1995). We propose that BDNF rescues neurofilament loss and hence hippocampal shrinkage.

It is further suggested, that DCS may mediate its effects through either distinct or overlapping (with fear conditioning) NMDAR-activated signal transduction cascades. One of these downstream effectors, BDNF, was found to be up regulated by DCS treatment. This DCS-induced BDNF increase is in line with reports showing activation of the NMDAR leading to

increased BDNF expression (Marini et al., 1998; Yaka et al., 2003). Furthermore, this finding suggests that DCS might act via BDNF and its downstream signalling pathways in enhancing fear extinction. Given that BDNF signalling was shown to facilitate LTP (Kang & Schuman, 1996; Nagappan & Lu., 2005), we hypothesize that the modulation of this neurotrophin by DCS during fear extinction might increase the growth and differentiation of DHC neurons and thus strengthen their connections resulting in greater enhancement of fear extinction. Changes in BDNF levels, due to pre-extinction DCS treatment, mirrored the improvement of PTSD-like symptoms noted in our behavioural results of DCS treated rats i.e. less freezing behaviour and anxiety-like symptoms.

Neuronal nitric oxide synthase was also found to be down regulated in response to DCS treatment. The above finding suggests that DCS decreases oxidative stress by either enhancing antioxidant proteins or reducing free radical production. Indeed the current study demonstrated a simultaneous decline in apoptotic initiator and oxidative stress proteins, and an increase in antioxidant proteins. The present findings of DCS treatment on fear conditioned rats may reflect the possible neuroprotective effect of DCS on increased NMDAR activation and neurotoxicity following fear conditioning. Previous studies have demonstrated the counteractive effects of DCS on NMDAR overstimulation, acting as a possible neuroprotective mechanism (Naskar & Dreyer, 2001).

Collectively, the data here demonstrates that a specific neurochemical process and different molecular signalling pathways underlie hippocampal differences between fear conditioned and fear extinguished rats. Like conditioning, fear extinction was shown to be dependent on NMDAR, CaMKII, MAPK activation (Kanterewicz et al., 2000). Neurochemical and proteomic analyses of the DCS treated-fear extinguished rats, demonstrated that the activation of the NMDAR triggered downstream proteins and signalling cascades involving CaMKII and MAPK.

Given that these molecules and their pathways underlie LTP and were expressed in our fear extinguished animals, we conclude that fear extinction indeed involves a form of learning.

Noteworthy is the difference in molecular effects of fear conditioning on the two age groups of rats. We propose neuromolecular sequelae of adolescent acute trauma include the following proteins: tweety, tubulin and beta adducin. While the neuromolecular sequelae of adult acute trauma include T-complex, hexokinase, cytochrome c, hippocalcin and neural cell adhesion proteins. DCS-treated rats displayed a similar protein expression pattern when compared across age, strongly suggesting that the normal neurophysiology of adolescents is significantly different to that of adults. The abovementioned proteins are activated upon NMDAR stimulation and interact with nNOS and BDNF, thus their identification allows for molecular correlations to be made between NMDARs, nNOS, BDNF, fear conditioning and extinction.

A possible early molecular marker, neurabin-2 was found to be differentially expressed in PND 33 shocked rats. Interestingly, the protein neurabin-2 was only expressed in adolescent rats. Specifically, a significant increase was observed in neurabin-2 protein expression levels following the fear conditioning protocol of the present study. This age-specific expression of neurabin-2 in response to trauma suggests that an increase in this scaffolding protein may indicate anxiety-like symptoms in young adults. Since neurabin-2 has been implicated in excitatory synaptic transmission and given that neurabin-2 activity is known to be modulated by Ca^{2+} , the potential of this protein as a biomarker for PTSD in adolescents, justifies further investigation.

In summary, we showed that trauma led to a number of behavioural and cellular alterations. Shocked rats displayed anxious-like symptoms as well as a dysregulated NMDA- Ca^{2+} system that activated the ROS-pathway and ultimately manifested as aberrant behavioural responses.

In particular, alterations in molecular markers related to neuronal structure, plasticity, cell signalling, apoptosis and energy and oxidative metabolism were observed. These abnormalities are consistent with cellular evidence of hippocampal-behavioural pathology. The current study thus highlights the complexity of behavioural and cellular interactions and motivates the need for studying the role of multi parameters in pathologies such as anxiety disorders. Finally the study demonstrates that proteomic techniques are powerful tools to provide holistic information about disease pathology while simultaneously reveal novel biomarkers of disease states.

However, this study did present with a few limitations. For one, the ELISA-kit utilized for nNOS determination, measured the synthesis of this enzyme and not its activity. Therefore it is suggested that future studies should measure the levels of its substrate (e.g. L-arginine) or product (e.g. NO) to gain a better impression of its activity. Secondly, total NMDAR1 protein expression was measured which does not necessarily indicate changes in activity. This limitation can be addressed in future studies by measuring phosphorylation of NMDAR1 and/or electrophysiology of AMPA/NMDA receptors. Thirdly, verification of the differentially expressed hippocampal proteins is required to confirm the expression levels as observed. Future studies should therefore employ techniques such as Western Blotting or specific ELISA kits. Specifically, expression levels of neurabin-2, which was only expressed in fear conditioned adolescents of the present study, need to be verified with future studies. Given that a previous study found this scaffolding protein to be expressed in adult animals (Allen et al., 1997). The observed lack of neurabin-2 protein expression in the current adult group could be ascribed to a detection issue with the mass spectrometer i.e. the mass spectrometer might not have detected the protein in the adult sample.

We conclude that the overall data is evidence of the molecular efficacy of DCS as augmentation agent in enhancing fear extinction. Hence, we suggest that DCS may be used as effective treatment in conjunction with cognitive behavioural therapy for clinical PTSD. Furthermore, the present work points to the importance of considering the age factor when patients present with anxiety-related disorders such as PTSD.

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