

https://dspace.org/

Early and subsequent life stress: physiological responses, moderating events and outcome

Muller, Denise Margaret

2015-03-27
http://hdl.handle.net/10539/17354

Downloaded from a DSpace repository. https://dspace.org/

EARLY AND SUBSEQUENT LIFE STRESS: PHYSIOLOGICAL RESPONSES, MODERATING EVENTS AND OUTCOME.

Denise Margaret Muller

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, South Africa, 2014



DECLARATION

This thesis is submitted in the optional format, approved by the Faculty of Health Sciences, of published work and work submitted for publication with an encompassing introduction and conclusion.

I declare that the work contained in this thesis is my own, except where acknowledged as otherwise.

This work has not been submitted for a degree at any other university.

_____

Signed on the _____ day of _____, 2014

ETHICS APPROVAL

Human

Adhering to the principles of the Declaration of Helsinki, the University of the Witwatersrand's Human Research Ethics Committee approved all work with the children from The Teddy Bear Clinic (M070724 and M060250) and the control group of children (M10635). See Appendix A.

Animal

The University of the Witwatersrand Animal Ethics Screening Committee approved all procedures performed in my animal experiments (2005/74/6). I have included a copy of all ethics clearance certificates in Appendix A.

ABSTRACT

- 54 225: crimes against children reported in 2010/2011.
- 28 128: sexual offences
- 30% of the sexual offences occurred in children younger than ten years.

These figures are a stark reminder of the growing number of children who experience deprivation, abuse and maltreatment in South Africa (Unicef, 2013). Although controversy exists with the reporting methods and the accuracy of recall in adult patients, it remains evident that a significant number of female children are sexually abused. Associations between early life stress and later life dysregulation of the hypothalamic-pituitary-adrenal axis hormone, cortisol, and the immune system cytokine, interleukin 6, have been found in adult patients and in animal studies. Importantly, although there is also evidence that early life stress results in later life neurobiological changes, we have to date, no identifiable biological markers to assist with diagnosis or to inform treatment strategies in young children who present with early life stress such as sexual abuse or maternal neglect.

Thus, there is a growing imperative to establish whether the potential precursor biomarkers are evident in early in development following adverse life conditions. Therefore the research focus of the thesis was to investigate (1) whether dysregulation of the HPA axis is evident in young children who are exposed to the traumatic stress of abuse, (2) whether there is evidence that inadequate maternal care, during the neonatal stage of development, has an impact of HPA and immune function and consequently on

body mass and finally, whether there evidence of HPA axis and immune dysregulation occurring during subsequent stress responses during adolescence?

Two groups of young girls between 6-12 years old who presented to a sexual abuse forensic clinic were found to have significantly different circulating cortisol stress responses to the forensic examination. Importantly, children who had experienced other life stressors had an attenuated cortisol response to the forensic examination. Thus, given these results which indicated that other life stressors such as maternal care may have an influence on cortisol responses to a stressor, two pre-clinical animal studies were implemented to investigate the impact of maternal deprivation. Good maternal attachment has long been considered by many psychologists to provide children with ameliorating benefits that help prevent the long term effects of early life trauma on mental and physical health.

Extensive research using animal models of maternal deprivation has delineated clear evidence that inadequate maternal care has an impact on the HPA axis and the immune system. In a pre-clinical rodent model of early life stress, a relationship between maternal deprivation and the biomarkers of stress, cortisol, and inflammation, IL-6, and visceral fat mass associated with an increased body mass were identifiable at an early developmental time point namely, on the day of weaning and following the stress of weaning, twenty-four hours later. Specifically, maternal deprivation into the third week of neonatal life results in an attenuation of corticosterone following a second early life stressor namely, weaning. Moreover, in the juvenile rats an increase in body mass and

visceral fat mass is evident in the rats that had experienced maternal deprivation. An important finding of the pre-clinical research was the finding of an increase in visceral fat mass and body mass. Thus, this research provides evidence for the occurrence of early physiological dysregulation following maternal deprivation. Critically, this research has also raised vital and intriguing questions regarding the type of stress experienced, moderating factors and other potential circulating biomarkers.

Overall these findings have implications for the establishment of effective diagnostic and treatment programs and consequently for recovery and prevention of long-term psychiatric and medical sequelae. Moreover, it is possible that this research will enable the implementation of a prospective study of circulating endocrine biomarkers in sexually abused children. Based on the stress response in the children from the clinic we would recommend that cortisol reactivity be the basis of clinical stratification of a response to a stressor, either hypercortisolism or hypocortisolism, should be considered when evaluating children who have been sexually abused.

With respect to the overarching goal of this research, to investigate peripheral blood biomarkers for early-detection of distress in children who have been sexually abused, there is evidence for dysregulation in glucocorticoid responses in sexually abused children. Moreover, in an animal model of maternal deprivation a similar pattern exists in response to a stressor. Additionally, in light of the increasing obesity epidemic, the finding in the animal model of early life stress, of increased body mass and visceral fat mass merits further investigation.

DISCLAIMER

My PhD thesis is designed as stand-alone manuscripts. These manuscripts have either been submitted for publication or have already been accepted for publication. As a consequence there may be some repetition between chapters. Furthermore, although there is some duplication of methodologies, the methods specific to each chapter are presented prior to the results in each chapter.

RESEARCH OUTPUTS ACCEPTED FOR PUBLICATION

Chapter 2

Muller, D.M., Errington, S., Szabo C.P., Pitts, N., & Jacklin, L. (2014). Cortisol and IL-6 Responses to Stress in Female Children Presenting at a Sexual Abuse Clinic. Journal of Child & Adolescent Trauma,

Chapter 3

Muller, D.M., Errington, S., Szabo, C.P., Pitts, N., & Jacklin, L. (2014) Disparate plasma cortisol concentrations in sexually abused female children from Johannesburg, South Africa. Child Abuse and Neglect.

RESEARCH OUTPUTS SUBMITTED FOR PEER REVIEW

Chapter 4

Muller, D.M., Szabo, C.P., & Pitts, N. (2014) Variation of maternal deprivation protocol reveals increased body mass and visceral fat mass in female Sprague Dawley rat pups. Stress.

DECLARATION AND ACKNOWLEDGEMENT OF THE CONTRIBUTIONS OF CO-AUTHORS.

As part of the Declaration, co-author contributions for each of the chapters are detailed below:

Chapter 2:

Muller, D., Errington, S., Szabo, C.P., Pitts, N., & Jacklin, L. (2014). Cortisol and IL-6 Responses to Stress in Female Children Presenting at a Sexual Abuse Clinic. Submitted to Journal of Child and Adolescent Trauma.

The idea for this study was my own, developed in association with Professor Lorna Jacklin and Sheri-Lee Errington of the Teddy Bear Clinic. I prepared the documentation, collected and analysed the data, and wrote the manuscript. Dr Pitts assisted with cortisol and cytokine assays and the interpretation of the data. Dr Pitts, Sheri-lee Errington, Professor Szabo and Dr Jacklin reviewed the manuscript prior to submission to the journal.

Chapter 3

Muller, D., Errington, S., Szabo, C.P., Pitts, N., & Jacklin, L. (2014). Disparate plasma cortisol concentrations in sexually abused female children from Johannesburg, South Africa. Accepted by Child Abuse and Neglect.

The idea for this study was my own, with approval from with Professor Lorna Jacklin and Sheri-Lee Errington of the Teddy Bear Clinic and developed with my supervisor Dr Neville Pitts. I prepared the equipment, collected and analysed the data, and wrote the manuscript. Dr Pitts assisted with sample collection, RIA assays and interpretation of the data. Dr Pitts, Professor Szabo and Dr Jacklin reviewed and edited the manuscript.

Chapter 4

Muller, D., Szabo, C.P., & Pitts, N. (2014). Variation of maternal deprivation protocol reveals increased body mass and visceral fat mass in female Sprague Dawley rat pups. Submitted to Comparative Psychology.

The idea for this study was my own, developed with my supervisor Dr Neville Pitts. I prepared the equipment, collected and analysed the data, and wrote the manuscript. Dr Pitts assisted with sample collection and the assays. Dr Neville Pitts helped with interpretation of the data and reviewed the manuscript. Professor Szabo reviewed and assisted with editing the manuscript.

Chapter 5

Muller, D., Moodley, A., Dukhan, T., Kock, M., Szabo, C.P., & Pitts, N. (2014). Increased visceral obesity in juvenile female Sprague-Dawley rats following cumulative life stressors: Implications for future research.

The idea for Phase 1 was my own, developed with my supervisor Dr Neville Pitts. I prepared the equipment, collected and analysed the data, and wrote the manuscript. Logistically the study included two phases thus we were able to included additional data collection due to insignificant differences between the groups. Thus, with assistance from Dr Pitts, we redesigned the data collection in the second phase. Phase 2 was also undertaken in collaboration our Honours students, Tanusha Dukhan, Melissa Kock and Aaisha Moodley, who assisted with data collection. Dr Pitts assisted with sample collection and the assays, helped with interpretation of the data and reviewed the manuscript. Professor Szabo reviewed and assisted with editing the manuscript.

CONFERENCE PRESENTATIONS

International

Muller, D., Worsley, C., Errington, S., Pitts, N., Suchard, M., Szabo, C.P., & Jacklin, L. Pilot study to correlate circulating cytokine and cortisol levels in South African children presenting acutely to a sexual abuse clinic. Poster. 9th Biological Psychiatry Congress, Paris, France, 28 June - 2 July 2009.

Muller, D., Worsley, C., Errington, S., Pitts, N., Suchard, M., Szabo, C.P., & Jacklin. L. Endocrine and cytokine profiles in South African children presenting at Johannesburg's Teddy Bear Clinic: Implications for diagnosis and treatment. Oral. ISPCAN, Honolulu, Hawai'i, September 26 – 29, 2010.

Muller, D., Szabo, C.P., & Pitts, N. Disrupted attachment: Allostatic load one day post weaning. Poster. 18th Annual PNIRS Meeting, Chicago, Illinois, June 8-11, 2011.

Local

Muller, D., Jacklin, L., Szabo, C.P. & Pitts, N. Impact of early life stress on the immune and endocrine systems: Lessons from an animal model. 15th South African Psychology Congress, Cape Town, South Africa, 12-14 August 2009.

Muller, D., Worsley, C., Errington, S., Pitts, N., Suchard, M., Szabo, C.P., & Jacklin, L. Correlation of circulating cytokine and cortisol levels of children presenting at the

Teddy Bear abuse clinic. Cross Faculty Post graduate Symposium, University of Witwatersrand, Johannesburg, 20 – 21st October 2009.

Muller, D., Worsley, C., Errington, S., Pitts, N., Suchard, M., Szabo, C.P., & Jacklin, L. Endocrine and cytokine profiles in South African children presenting at Johannesburg's Teddy Bear Clinic. Implications for diagnosis and treatment. Oral. SA CAPAP, Johannesburg, South Africa, July 26 – 29, 2011.

Muller, D., Jacklin, L., Szabo, C.P. & Pitts, N. Altered biological responses following childhood neglect and abuse: Implications for future health. Oral, IACAPAP 2014, Durban International Convention Centre, August 11-15, 2014.

DEDICATION

First and foremost, I would like to dedicate this work to my late Mom and Dad. Lifetime stressors marked the landscape that they grew up in. The lifelong impact on both soma and mind of the early stress that each of them endured instilled in me a desire to decipher and understand it. I would like to acknowledge my deep and heartfelt gratitude to the love and care that they gave me. From backgrounds that were very challenging they rose above to offer me a better life.

Secondly, I dedicate this thesis to my best friend Angus Grimbeck who died from a ruptured aortic aneurysm at the end of 2007. He was my dearest friend and confidant who provided unstinting support to me especially in the early days of my PhD endeavor.

Although the road towards completion of my PhD has been long, precipitous and fraught with significant obstacles due to the death of both my mother, Christina Muller, and my best friend, Angus Grimbeck during the PhD journey, their support and love have sustained me.

Finally, it is my hope that this thesis and the work that has emanated from it, inspires more research into the physiological impact of abuse on young children.

ACKNOWLEDGEMENTS

Dr Neville Pitts

"What is the question?" This question from my primary supervisor, Dr Neville Pitts has been a key factor that has allowed me to understand scientific thought and writing. The question, the processes that I followed to answer the question and the resultant answer has enable me to direct this thesis from being a psychological debate to being about answering a specific question. Further, I would like to thank him for his keen interest in the area of stress and his unwavering interest in the literature and the latest developments in the field of stress. Given our extremely different backgrounds viz. scientist and psychologist, I have most certainly presented him with challenge while I learnt to focus on the scientific question.

Professor Christopher Szabo

Professor Szabo has to be credited for his ability to succinctly enable me to develop the title for this thesis based on the ideas that I had presented to him. In some ways, during the data collection and laboratory work, he was in the background but when I really needed his support during the difficult years after my friend had died, he offered unwavering and emotionally coherent support. I would like to thank him wholeheartedly for providing that support and allowing me the time to heal and get back on track. I would also like to thank him for his insightful review of all the documentation that I have submitted to him.

Professor Lorna Jacklin

As an older student, it was extremely difficult to get funding for some of the scientific analyses. Professor Jacklin welcomed me to her department research meetings and encouraged ideas for research at the Teddy Bear Clinic. In particular, she enabled us to find the funding for the cytokine and cortisol determinations in the children from the Teddy Bear Clinic.

Sheri-Lee Errington

I would sincerely like to thank Sheri for her interest in the research, for always including me in the Teddy Bear research meetings and for her assistance with compiling the clinical information of the young patients of the Teddy Bear Clinic.

The Physiology Honours students

I would like to thank our Honours students Tanusha Dukhan, Aaisha Moodley and Melissa Kock for their work on the 2nd phase of the juvenile stress protocol. Under the supervision of both Dr Pitts and I, their work has greatly aided in allowing us to develop a clearer idea of the impact of a second stressor, foot-shock, on glucocorticoid responses and immune system responses. Importantly, following on from the Phase 1 evidence of increased body mass, their work highlighted the increase in visceral fat in stressed juvenile animals. I believe that the work we are doing in the Stresslab as a result of the Phase 2 protocol will yield significant insights into the role of maternal deprivation in the pathogenesis of obesity.

The children of the Teddy Bear Clinic and the bone density study

I would like to acknowledge and thank all the children and families that were part of this research. I believe that the knowledge I have gained will help some of my patients and the many parents that I encounter as part of my clinical psychology practice.

Central Animal Service and the animals

Finally, acknowledgement has to be given to the staff of the Central Animal Service (CAS) for all their assistance with the rats that were part of this study. Although I took over all husbandry duties of the rats during the actual study, the CAS staff were always supportive and available to assist.

TABLE OF CONTENTS

DECLARATION II

ETHICS APPROVAL III

Human iii

Animal iii

DECLARATION AND ACKNOWLEDGEMENT OF THE CONTRIBUTIONS OF CO-AUTHORS. IX

ABSTRACT IIV

DISCLAIMER VII

RESEARCH OUTPUTS ACCEPTED FOR PUBLICATION VIII

RESEARCH OUTPUTS SUBMITTED FOR PEER REVIEW VIII

DECLARATION IX

CONFERENCE PRESENTATIONS XII

International xii

Local xii

DEDICATION XIV

ACKNOWLEDGEMENTS XV

TABLE OF CONTENTS XVIII

LIST OF FIGURES XXI

LIST OF TABLES XXIII

LIST OF ABBREVIATIONS XXIV

GLOSSARY XXV

PREFACE XXX

CHAPTER: INTRODUCTION 1

CHAPTER 2: Cortisol and IL-6 responses to stress in female children presenting at a sexual abuse clinic. 69

CHAPTER 3: Disparate plasma cortisol concentrations in sexually abused female children from Johannesburg, South Africa. 87

CHAPTER 4: Variation of maternal deprivation protocol reveals increased body mass and visceral fat mass in female sprague dawley rat pups. 105

CHAPTER 5: Increased visceral obesity in juvenile female sprague-dawley rats following cumulative life stressors: implications for future research. 132

CHAPTER 6: Summary of the critical human and animal findings following early life stress. 164

CHAPTER 7: CONCLUSIONS 174

CHAPTER 8: REFERENCES 182

APPENDIX A 236

ETHICS APPROVALS 236

APPENDIX B 241

COPYRIGHT APPROVALS 241

LIST OF FIGURES

CHAPTER 1

Figure 1. HPA axis stress response. 18

Figure 2. Allostatic load. 21

Figure 3. Figure 3. Remodelling of brain under stress. 27

Figure 4. Glucocorticoid receptor (GR). 31

Figure 5. Circadian rhythms. 35

Figure 6. Adiposity and inflammation. 55

CHAPTER 2

Figure 2. Morning plasma cortisol concentration. 78

Figure 2. Correlation of IL-6 to Cortisol. 79

CHAPTER 3

Figure 1. Mean plasma cortisol concentration. 96

CHAPTER 4

Figure 1. Plasma corticosterone concentrations. 115

Figure 2. Plasma IL-6 concentrations. 116

Figure 3. White blood cell counts. 117

Figure 4. Body Mass. 118

Figure 5. Visceral fat mass. 119

Figure 6. Linear regression between corticosterone and body mass. 121

Figure 7. Linear regression between IL-6 and body mass. 122

Figure 8. Linear regression, on the day of weaning, between body mass and white cell count. 123

CHAPTER 5

Figure 1. Experimental design. 140

Figure 2. Plasma corticosterone concentrations. 146

Figure 3. Plasma IL-6 concentrations. 147

Figure 4. Body Mass. 148

Figure 5. Percentage visceral fat. 149

Figure 6. White blood cell counts. 150

Figure 7. Corticosterone metabolite concentrations in faecal samples. 153

LIST OF TABLES

CHAPTER 2

Table 1. Demographics

Demographic of pilot group of children presenting to the Teddy Bear forensic clinic in a public hospital in Johannesburg, South Africa. 77

CHAPTER 3

Table 1. Demographics

Demographics of children presenting to the Teddy Bear forensic clinic in a public hospital in Johannesburg and control group undergoing a bone density scan. 95

CHAPTER 5.

Table 1 Daily changes in FGM secretion

Daily changes in FGM secretion following cage switches and foot-shock. Graphical representation of FGM changes for all groups are presented in Figure 7. 152

LIST OF ABBREVIATIONS

5-HT	serotonin
ACTH	adrenocorticotropic hormone
BDI	Beck Depression Inventory
BPD	Borderline Personality Disorder
CAR	cortisol awakening response
CM	childhood maltreatment
CRH	corticotropin releasing hormone
CRP	C-Reactive Protein
CSA	childhood sexual abuse
CVD	cardiovascular disease
DSM-5	Diagnostics and statistics manual version five
ICU	Intensive care unit
IL-1α	interleukin-one alpha
IL-1β	interleukin-one beta
IL-6	interleukin-six
MDD	Major depressive disorder
PBMC	peripheral blood mononuclear cells
PTSD	Post Traumatic Stress Disorder
SES	socio-economic status
TNF-α	tumour necrosis factor-alpha
TSST	Trier Social Stress Test
WHO	World Health Organization

GLOSSARY

Adrenocortiotropic hormone (ACTH) The stimulating peptide hormone released from the pituitary gland in response to the release of CRH from the hypothalamus.

Allostasis: Allostasis refers to the ability of an organism to maintain stability through physiological change. During stressful events there is a need for a rapid response by changing physiological variables such as cortisol secretion, heart rate, blood pressure and glucose availability (McEwen, 1998; Sterling, 2004).

Allostatic load Allostatic load occurs when the organism demonstrates lack of behavioural, psychological and physiological adaptation to a stressful event (McEwen, 1998).

Altricial Altricial refers to the need for a young neonatal animal to be nursed or nourished by a care giver, usually the mother, for a long period to survive.

Anxiety A number of categories of anxiety are defined in the 5th edition of the Diagnostic and Statistics Manual (DSM 5; APA, 2013). Briefly, a generalized anxiety disorder occurs when the patient has persistent, excessive, and unrealistic concerns about everyday occurrences.

Attachment Attachment, a psychological term, denoting the connection that an infant has to caregivers (Bowlby, 1987a; 1987b). Scientifically, attachment has been operationalized as maternal deprivation (Levine, 1957).

Biomarkers Biomarkers are biological variables of physiological functioning that

	are used to evaluate whether a patient has a normal response to a particular manipulation for example, in evaluation of a condition such as diabetes mellitus both glucose and insulin are the biological markers. With respect to the current work, the biological markers under investigation are cortisol and the immune system cytokine IL-6.
Borderline Personality Disorder (BPD)	BPD is a psychiatric condition which consists of a variety of symptoms such as impaired mood regulation, unstable relationships with others, self-harming behaviors and impulsivity. Diagnosis is made on the basis of the patient fulfilling at least five specific criteria (APA, 2013).
Central	Mechanisms, processes and neurotransmitters in the central nervous system are frequently referred to with the abbreviated term – central or centrally
Clinical	Investigations with patients in either a hospital or out-patient clinic.
Circadian	A biological endogenous process that oscillates between a zenith and a nadir level in a 24 hour night and day period. A circadian cycle may not be exactly 24 hours.
Corticotropin-releasing hormone (CRH)	In response to a stressor, physiological, psychological or environmental, the peptide hormone CRH is released from the hypothalamus to stimulate the pituitary gland to produce and release ACTH.
Cortisol (corticosterone n rodents)	A glucocorticoid steroid hormone released in a normal circadian rhythm with bolus secretions in response to a stressor.

Cytokines	Small peptide messengers of the immune system that travel in the blood stream to remote sites such as the brain or other tissues to activate or inhibit the immune system.
Depression	Depression or Major Depressive Disorder (MDD), a DSM-5 diagnostic category (APA, 2013), refers to a significantly low mood for a period longer than 2 weeks. At least 5 specific symptoms have to be present every day**.**
Dexamethasone suppression test	Dexamethasone, an exogenous glucocorticoid, simulates cortisol concentrations in the body. By administering dexamethasone, it is possible to evaluate the feedback mechanism from cortisol to the pituitary gland to evaluate ACTH production (see Introduction, Figure 1).
Diseases of lifestyle	Lifestyle diseases are diseases that are considered to occur due to effects from diet, environment, and lifestyle. Diseases such as heart disease, stroke, obesity, and diabetes type II are considered to be diseases of lifestyle.
Diurnal rhythm	Pertains to the daylight hours with the pattern repeated every 24 hours.
Dysregulation	A prolonged change away from an accepted normal range. For example, significantly low concentrations at a time of day when higher concentrations are expected. See HPA axis and diurnal rhythm.
Homeostasis	Homeostasis is a mechanism to ensure that any particular system in the body is regulated and maintained within a narrow range. For example, blood pH is maintained and regulated to remain within an extremely

	small range. Homeostasis may be contrasted with allostasis which allows a particular system such as the stress mechanism to adapt to changing environmental or physiological demands and then to return to a normal baseline value (see Allostasis).
HPA axis	The HPA axis comprises the neurotransmitters, CRH from the hypothalamus, ACTH from the pituitary and cortisol from the adrenal gland. The HPA axis is the major neuro-endocrine axis involved in modulating and mediating long term responses to stressful stimuli.
Maternal Deprivation	Seymour Levine (1957) operationalized the definition of maternal deprivation, initially coined by Bowlby, into biological investigations into the impact of the stress of maternal loss on the stress responses in infant animals.
Metabolic Syndrome	Metabolic syndrome is comprised of a cluster of known diseases namely, obesity, diabetes mellitus type II, hyperlipidaemia, high cholesterol and hypertension.
PBMC	PBMC's are white blood cells with a round nucleus as opposed to other white blood cells which have a lobed nucleus. PBMC's are critical components in the innate immune system to fight infection. Examples include the white blood cells, lymphocytes, monocytes and macrophages.
Personality Disorder	Personality disorders are a category of Section II in the DSM 5 (APA, 2013). A patient with a personality disorder usually presents with divergent patterns of behaviour, cognition and inner experience with

	respect to their cultural norms. Moreover, patients or close family member of patients with personality disorders usually experience significant distress and disability in everyday life.
Pre-clinical	Laboratory investigation using comparative animal species in order to identify biological markers that can be further investigated in a clinical setting.
Post -Traumatic Stress Disorder (PTSD)	PTSD is a disorder that may occur following a severely traumatic event. The disorder is characterized by flashbacks of the trauma, emotional blunting or hyperarousal, fear, avoidance of memories or of the place where the trauma occurred for more than a month after the traumatic event.
Stress	Stress, in the context of this thesis, is any event experienced by both animals and humans that produces significant lasting change in the physiological functioning of the individual organism
Telomere	Replication and stability of chromosomes is provided for by telomeres. Telomeres are the region of DNA at the molecular end of a linear chromosome. The functional base sequence of TTAGGG bases in the telomere is highly conserved.
Trier Social Stress Test	A laboratory test designed to induce stress in human participants when asked to speak to a unfamiliar and unfriendly group (Allen *et al.*, 2014).

PREFACE

"There are a thousand hacking at the branches of evil to one who is striking at the root." Henry Thoreau

The impact of child abuse, specifically in relation to the aetiology of the psychiatric diagnosis, Borderline Personality Disorder (BPD), has been a lifelong interest of mine. In 1993, at the start of my Master's Degree in Clinical Psychology, there was very little acceptance in South African psychological circles of the hypothesis that abuse and early life stress resulted in physiological perturbations in adulthood. As a Clinical Masters psychology student my impression was that the overriding paradigm in South Africa at that time was that young children with conduct disorders and adult patients, who retrospectively reported abuse and maltreatment, were fabricating their histories to receive attention. The primary sentiment at the time was that these were just "bad children, no wonder they were abused because no one could control them". In contrast, researchers such as Briere (1992), Finkelhor (1988a, 1988b) and van der Kolk (1987) were documenting the burgeoning evidence that adult patients with diagnosable Borderline Personality Disorder retrospectively reported severe early life stressors.

Today, in South Africa, childhood trauma and abuse are at epidemic proportions. In the 2010/2011 reporting year, UNICEF (2013) reported that of the 28 128 sex crimes reported approximately 30% of these occurred in children younger than 10 years of age. However, of concern, about the figures of sexual abuse from Unicef (2013), is that other forms of early life stress such as abandonment, death of a parent, emotional and verbal abuse are not considered. The combination of a variety of stressors is considered, by

many mental health practitioners, to result in complex trauma symptomatology suffused with pathological attachments to parental figures (D'Andrea *et al.*, 2012; Fonagy & Luyten, 2009). In essence, the primary element of good maternal attachment is considered to provide children with ameliorating benefits and resilience that helps prevent the long term effects of future trauma on mental and physical health (Tang *et al.*, 2013). Psychological therapy with both children and survivor adults has traditionally focused on exploring the emotional impact and behavioural ramifications that the abuse has on daily functioning. However, a specific focus on psychological perturbations has, in effect, maintained the Descartesian mind-body split (Greer, 2003). In contrast, recent research into the physiological and neurobiological changes that occur following early life stress has revealed that a significant organic component may be associated with the consequent problems encountered by survivors of adverse life experiences. Thus, biological changes occurring peripherally in the stress and immune systems may be evident behaviourally as neurobiological remodeling occurs (Schore, 2003).

A number of conceptual frameworks, theories and paradigms exist to explain the development of Borderline Personality Disorder (BPD). BPD is a complex admixture of brain and body chemistry, developmental stages, thought processes and affective states. Each has been the focus of entirely diverse areas of mental and physical health care. I envision a time when we will have a holistic view of the BPD patient, where the fear of medicalization will dissipate and we will be able to help our patients without fearing that they will lose their experience and their world view.

Thus, my thesis represents the integration and synthesis of my long standing belief that early life stress alters children physiologically. Moreover, patients attempt to verbalize these physiological alterations in their personal narrative. I believe that we are at the beginning of a very exciting journey that will begin to delineate the impact of a variety of stressors on different physiological processes in children. Investigations into potential biomarkers may, I believe, allow us the possibility of early diagnoses for all types of immunological deficits. Central to my belief of physiological perturbation is the impact that early life stress has on developmental process. Furthermore, the impact of stress on the HPA axis and the resultant secretion of the stress hormone, cortisol has a marked impact on the immune system.

However, notwithstanding all the scientific research into the impact of life stressors on the neurobiology of a developing altricial animal, the divide between psychology and biology remains. The discourse in both fields continues to maintain the divide with terminology specific to each discipline and thus the barrier is maintained. The need for a bridge across the divide is the reason for this thesis. In the field of child abuse there is a significant body of work on both sides of the divide with a growing need to understand the impact that life stressors have on the biology of an individual.

Thus, my PhD is predicated on the fact that the inescapable maltreatment of children in South Africa has reached epidemic proportions, with an excess of 1000 reports per month of children being sexually molested. Unfortunately the high number of reported abuse cases frequently does not include children that are abandoned, subjected to

maternal deprivation, emotional and verbal maltreatment or traumatised in any other way such as a death in the family. Thus, it is imperative that the future mental and somatic health of our patients requires an integration of the art of psychotherapy with the accumulating scientific knowledge regarding lifelong impact that early life stress has on the developing organism both centrally and peripherally. Scientific knowledge, over the last 20 years, has noted significant physiological impacts following early life stressors. However, the field of psychology has been slow to incorporate these findings into mainstream psychotherapy. Moreover, as a Clinical Psychologist, a major aim of my thesis is the integration of both a physiological and a psychological approach to allow for an increased clinical understanding of the biological impact of early life abuse on the development of later life psychopathology and somatic illness.

CHAPTER 1

INTRODUCTION

Early life stress in the form of inadequate maternal care and childhood maltreatment, are associated with the development of later life psychopathology (Belsky *et al.*, 2012; Tramantano *et al.*, 2003; Zanarini *et al.*, 1997). Psychiatric disorders such as Post-Traumatic Stress Disorder (PTSD; Daskalakis *et al.*, 2013b; Jovanovic *et.al*., 2009), anxiety and Major Depressive Disorder (MDD; Hayley *et al.*, 2005; Heim *et al.*, 2008, 2010) are associated with childhood adversity. Significantly, the psychiatric disorder Borderline Personality Disorder (BPD) is strongly associated with early life stress and sexual abuse (Belsky *et al.*, 2012; Briere & Runtz, 1987, 1988; Paris, 1993; Zanarini *et al.*, 2002).

The psycho-social determinants of BPD have been extensively theorized and are considered to be due to a combination of genetic predisposition (Gunderson & Lyons-Ruth, 2011), disruption of the early maternal-child relationship (Tramantano *et al.*, 2003) and exposure to early life stress in the form of physical, sexual and interpersonal trauma (D'Andrea *et al.*, 2012; Herman *et al.*, 1989). The proposed and recently implemented changes in the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5; American Psychiatric Association (APA), 2013), from a multiaxial system in the previous version, has seen a renewed focus on understanding the biological aetioligy of BPD. The complex inter-relationship between behavioural, emotional, social and personality features of BPD (DSM-5, APA 2013) may have resulted in relatively fewer investigations into the biological impact of sexual abuse in childhood (Wingenfeld et al., 2010). A renewed focus on evaluating trauma and biological perturbations in the aetiology of BPD has been evident in the last two decades

(Paris, 1993). Although all psychiatric disorders now appear in Section II of the DSM-5 (APA, 2013) no clear objective evidence has been established regarding the biological aetiology that early life stress may have on personality development (Kapur *et al.*, 2012). However, biological changes have been noted in adult patients diagnosed with BPD. The dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis and diurnal alterations in the circulation of the immune system messenger interleukin-6 (Kahl *et al.*, 2006) are associated with the retrospective recall of early life maltreatment in patients diagnosed with BPD. The relationship between early life abuse and consequent physiological perturbations to the HPA axis and the immune system in sexually abused children remains under-investigated. Moreover, the confounding factors associated with early childhood maltreatment do not always appear to induce significant personality changes in all children exposed to maltreatment (Akers *et al.*, 2006). Stone (1993) suggests that biological risk factors need to be evaluated with respect to their interaction with other risk factors such as maternal care and associated traumatic experiences. Thus, it is possible that the interrelationship between stress, developmental age and differential HPA and immune responses cause or moderates the development of long term detrimental outcomes (Akers *et al.*, 2006; Ford *et al.*, 2013).

Although a stress response with an increase in the stress hormones adrenaline and cortisol is beneficial and enhances performance (Akirav *et al.*, 2003; Crum *et al.*, 2013; Korte *et al.*, 2005; McEwen, 2006), a significant lasting change in the physiological response to stress, namely hyper-responsiveness or attenuation, is considered to be a dysregulated response to a stressor (see Figure 2; Logan & Barksdale, 2008; McEwen,

2000, 2006; Turnbull & Rivier, 1999). Extensive research on the physiological effects of severe stress, in animals and humans, has shown that both endocrine activation and altered immune responses may result in deleterious effects on physiological functioning and neurobiology (Holscher, 1999; McEwen, 2000; Rinne *et al.*, 2003).

As will be discussed in more detail in Section 1.2 the physiological imbalance caused by early life stress may be responsible for an increase in vulnerability to later psychopathology in humans, particularly following a severe stressor in later life (Goodman *et al.*, 2004; McEwen, 2000). To date, it is clear from a large portion of child abuse literature that the focus has primarily been on divergent behavioural manifestations of distress, with a paucity of information regarding the physiological mechanisms that underlie the behaviour. A factor lacking from the work undertaken to date is that we are as yet unable to utilize circulating plasma biological markers to reliably identify and thus consider intervention in vulnerable children when they first present to medical personnel (Hayashi-Takagi *et al.*, 2013).

The focus of this PhD is to investigate whether dysregulation of the biological markers cortisol and IL-6, associated with lasting neurobiological and physiological change, occurs in young female children patients who present for treatment following sexual abuse. As the timing and the severity of early life stressors may have a differential impact on later life outcomes (Johnson *et al.*, 2003), it is important to note that the terms "childhood abuse" and "lasting effects" imply a developmental perspective. There is also an imperative to investigate whether earlier life stressors such as maternal

deprivation may have a cumulative impact on the biomarkers cortisol and IL-6. In order to investigate the impact of stress in the neonatal stage of development, the current work also focused on developing an applicable maternal deprivation model to evaluate the lasting impact of a maternal deprivation in another altricial mammal, the rat.

This introductory chapter is thus structured as follows: firstly, a short exploration of the theoretical, behavioural and psychological understanding of the impact of stress in two developmental stages, namely neonatal and childhood. Secondly, a review the physiological processes and mechanisms, from both pre-clinical and clinical studies, that have been associated with stress in each of the abovementioned developmental stages. Thirdly, a review of the current knowledge of psychiatric and somatic sequelae, co-morbidly associated with early life stress, physiological dysregulation and the development of BPD. Fourthly, a review of possible moderating factors of physiological dysregulation is considered. Finally, the hypotheses and aims of the research are presented together with an overview of the focus in the succeeding chapters.

1. **Psychological and physiological research into the lasting impact of early and subsequent lifetime stress**

Child maltreatment is a problem in many countries of the world. Children are abandoned, forced into slavery, orphaned and abused, with child labour persisting. However, the relationship between early childhood abuse and the development of long term psychiatric disorders has only been extensively investigated since the 1980's (Briere, 1992; Finkelhor, 1988; Heffernan & Cloitre, 2000). The theoretical premise of

the impact of early life stress was first introduced by Freud in 1897 (Boschan, 2008). Although Freud subsequently denounced trauma as an explanation for later life distress, a number of psychological theorists maintained that long term behavioural changes occur following early life adversity (Boschan, 2008; Bowlby, 1952). Over the last two decades maltreatment of children has been found to result in a number of adverse lifetime behavioural, psychological and health outcomes (Heim *et al.*, 2004, 2008; Hostinar *et al.*, 2013). In adulthood, those maltreated as children have been found to have an increased risk of psychiatric disorders such as MDD, anxiety and personality disorders (Cohen *et al.*, 2001; Heim *et al.*, 2008; Johnson *et al.*, 2000; Lee *et al.*, 2012), as well as substance abuse disorders (Lansford *et al.*, 2010). Furthermore, a link between early life stress and later life somatic disorders such as obesity (Hyland *et al.*, 2012; Torres & Nowson, 2007) and cardiovascular disease (Sansone *et al.*, 2011) has been established.

1.1 Psychological and behavioural descriptors of early life stress responses

In the psychological and psychiatric literature, a significant portion of the work on the response to stressors in childhood has focused on behavioural descriptors and their correlation with the subsequent development of specific psychiatric disorders (Tramantano *et al.*, 2003). In particular, there is significant evidence since the 1980's which links sexual abuse in childhood to the development of BPD (Herman *et al.*, 1989).

1.1.1 Maternal separation and deprivation

Unstable, intense relationship difficulties and the frantic efforts to avoid abandonment are key characteristic of patients who are diagnosed with BPD in adulthood (APA,

2013). These relationship and attachment difficulties are evident in more than 90% of adult BPD patients (Gunderson & Lyons-Ruth, 2008). Bowlby's (1952, 1987a) seminal work defined attachment behaviour as the relationship between the mother and the infant. Bowlby's (1952) theory of attachment and loss, and the consequent separation anxiety, evident on the loss of an infant's primary attachment, was developed as a direct result of a number of studies that were conducted in Britain during World War II and in the early 1950's. The common practice of separating young children from their mothers when they were hospitalised or when the mothers had another baby formed the basis of Bowlby's (1987a) attachment theory. In particular, the behavioural impact of early life distress following the loss of maternal care was extensively documented by Bowlby (1995) in his essay for the World Health Organization entitled "Maternal Care and Mental Health". Good maternal care is considered to provide the basis for lasting connectedness between individuals and also be an important factor in the modulation of affect and consequently a determinant of later life health or psychopathology (Belsky, 2001; Bowlby, 1987a; Dewitte *et al.*, 2010; Tang *et al.*, 2013).

Attachment to a caretaker appears to occur regardless of quality of the care, resulting in one of the defined styles of attachment and the strategies that an individual may employ to alleviate their emotional anxiety or distress (Bernard & Dozier, 2010; Breidenstine *et al.*, 2011; Dewitte *et al.*, 2010). Furthermore, secure attachments are positively related to appropriate exploratory behaviour in infants and young children who are able to explore their environment returning safely to adult caregivers if distressed (Zeanah *et. al.*, 2011). In contrast maternal deprivation may lead to anxiety in relationships

(Breidenstine *et al.*, 2011). Based on the level of anxiety and avoidance that a person may experience in a relationship, four prototypical behavioural styles of attachment have been categorized namely, secure, avoidant, ambivalent/resistant or disorganized (Ainsworth, 1985; Dewitte *et al.*, 2010). Attachment difficulties have been reported in patients diagnosed with BPD, MDD or anxiety who retrospectively report childhood neglect and maltreatment (Nader, 2011; Paris & Zweig-Frank, 1993). Insecure attachments are currently determined by behavioural, subjective and retrospective reporting of an individual's emotional response to questions regarding their maternal relationships and their current relationships (Dewitte *et al.*, 2010). Moreover, good maternal care and secure attachments are recognized as moderating factors against future traumatic exposure (Breidenstine *et al.*, 2011; Finkelhor, *et al.*, 2011; Nader, 2011). Bowlby (1952) proposed that a good maternal relationship was needed to provide children with ameliorating benefits and resilience required to prevent the long term effects of later trauma on mental and physical health (Bowlby, 1952, 1987a, 1995). However, as noted by Dewitte *et al.* (2010) a more comprehensive measure of attachment needs to be established to include neuro-endocrine reactivity, namely measures of cortisol, self-reported distress in a relationship and behavioural indices.

1.1.2 Childhood and adolescent stressors

From the late 1980's there was a growing awareness among clinicians of the increasing numbers of children maltreated and neglected each year (Briere & Runtz, 1987, 1988). Briere (1992) categorised the long term impacts of abuse as either psychological, impaired self-reference, behavioural and relational. Childhood sexual abuse, one of the

most prominent stressors, has gained increased prominence with the occurrence of horrifying events in various countries such as South Africa in 2013. Mathews *et al.* (2013) report that 44.4% of all cases of sexual assault reported to the authorities in South Africa involve a child under the age of 17. Similarly, in the United States of America, Finkelhor *et al.*, (2013) in their updated review on violence experienced by children, note that a significant amount of crime and violence still occurs in the lives of children. Retrospective recall of early life sexual abuse for women is: 27% in the United States and 12% in Britain compared with a South African reported prevalence among white female university students ranging from 29-44% (Dawes *et al.*, 2007). A diagnosis of BPD is also associated with early life sexual abuse (Carvalho Fernando *et al.*, 2012; Heffernan, & Cloitre, 2000; Herman *et al.,* 1989; Rinne *et al.*, 2003).

The stress experienced by sexually abused children has been recognized as a significant factor for the development of later life problems (Finkelhor *et al.*, 2011; Sansone *et al.*, 2011; Zanarini *et al.*, 2002). It is evident that these figures may only represent the tip of the iceberg with respect to early life stressors that children face. Clinically it is evident that adult patients who present with BPD have experienced other early life stressors and also present with significant co-morbidity, with a range of the psychiatric disorders such as MDD and anxiety (Cohen, 2008; Kahl *et al.*, 2006; D'Andrea *et al.*, 2012; Sack *et al.*, 2012). The growing awareness that trauma in childhood and adolescence results in symptoms in adulthood, has called into question long cherished beliefs held about children's resilience in the face of trauma (Briere, 1992).

Trauma resulting in post-traumatic stress responses may easily have an impact on the child's subsequent psychological and social maturation (Briere, 1992), the effects of which are usually interactive and dynamic. Numerous psychological theorists have proposed developmental theories, based on behavioural and clinical evidence, to account for the impact that early life trauma has on the development of personality (Erikson, 1974, 1977; Freud, 1987; Kernberg, 1993; Klein, 1955; Mahler *et al.*, 1975; Winnicott, 1965, 1987, 1988; Belsky *et al.*, 2012). Briere (1992) detailed three distinct stages of adaptation to abuse (i) an initial response involving post-traumatic stress symptoms, negative affect and cognitive impairment, (ii) assimilation and adaptation of coping behaviours to increase security and decrease negative affect and finally (iii) long term elaboration and secondary accommodation reflecting the impact of the initial reaction and the personality adjustments to cope with the persistent dysphoria.

However, a significant disparity exists between the various behavioural and psychological characteristics and effects of abuse noted following early life sexual abuse. Controversy has remained regarding the aetiology of the long-term effects of childhood sexual abuse (Hendricks *et al.*, 1993; Finkelhor *et al.*, 2011). While negative life experiences and empathic maternal failures are implicated in the development of psychopathology, none of the above theories provide for a detailed consideration of the impact of trauma on the physiological and neurobiological impacts of early life stress. The trauma that an infant or child experiences occurs during critical stages in the development of affect regulation and of a sense of self (Schore, 2003). During the early developmental stages children validate their understanding of self, others and the world

around them (Schore, 2003). Victimization of a child and cumulative exposure to violence has a significant impact with the respect to the development of later life levels of depression and aggression. This victimization is a significant predictor of mental health (Turner *et al.*, 2006). Survivors of childhood sexual abuse (CSA) often present with heterogeneous, complex and diverse symptomatology. The result is a list of disparate adjectives that provide no clear guidelines regarding the risk for long term pathology which may become evident in any particular abused or stressed child. Thus, a significant aspect of adult psychopathology may be the result of dysregulation in stress responses following early childhood trauma. Moreover, the long term effects of stress may have neurobiological, and consequently, personality effects in some children and not others. Pathological and dysfunctional aspects noted in the clinical setting with adult patients may be the result of a strategy that the individual has developed to ameliorate the biological impact of the trauma.

1.2 Physiological responses to early life and subsequent stressors

A growing body of work on the biological implications of early life stress on both physiology and the developing brain has significant implications for the aetiology of BPD (Heim *et al.*, 2008; Irwin, 2008; Irwin & Miller, 2007; Levine, 2005; McEwen, 2003a; Wachs *et al.*, 2013). In recent years, the link between BPD, early life stress and HPA axis responses to stress has been demonstrated in a number of studies (Kahl *et al.*, 2006; Rinne *et al.*, 2002; 2003; Carvalho Fernando *et al.*, 2012). In particular, the long-term implications of maternal deprivation on the HPA axis when an altricial animal is exposed to a subsequent stressor have been extensively documented (Anisman *et al.*,

1998; Heim *et al.*, 2004; Levine, 1957; Levine, 2005; Lyons *et al.*, 2010; McEwen, 2006).

Stress, a ubiquitous factor of life, experienced throughout the life-span of any organism, results in a need to adapt to the environment to maintain homeostasis (McEwen, 2006). However, adverse early life stressful experiences have been associated with physiological effects, such as increased HPA activity and altered immune responses (Boscarino, 2004; Delahanty *et al.*, 2004, Maughan & Rutter, 1997; Rinne *et al.*, 2003) and consequent neurobiological changes (McEwen, 2006). The plasticity of the nervous system to a variety of life experiences has been implicated as a major factor in the development of neurobiological changes that occur following early life stress (Heim *et al.*, 2004; Wachs *et al.*, 2013). Critical periods of development have been associated with increased sensitivity to circulating stress hormones and immune system markers resulting in greater neurobiological impacts than at other developmental stages (Heim *et al.*, 2004). Notwithstanding the well documented long-term implications of neurobiological impact following early life stress, this invaluable knowledge does not provide clinical practitioners with applicable objective measures when parents, teachers or therapists identify behavioural evidence of stress in young children (Bernard & Dozier, 2010; Fries *et al.*, 2005). Therefore this section will focus on the pertinent research into the HPA axis and immune system dyregulation following early life adversity.

1.2.1 Biological investigations into early life stress

Although Bowlby (1952) speculated that physiological changes occurred during maternal deprivation, very little was known about the biological variables that were affected by early life distress. However, Bowlby considered the quality of early maternal care a critical component for future mental health and pivotal in the dynamic relationships that an individual forms in later life (Bowlby, 1952, 1987a; Dewitte *et. al*., 2010; Steele, 2010). The post-natal environment has been considered to have a significant contribution towards the alteration of endocrine, immune and neurobiological markers (Teicher, 2002). Research by Levine (1957) opened the way to examine the biological impact of maternal deprivation in pre-clinical animal models of early life stress. In rodent models of early life stress a period of time called the stress hyporesponsive period (SHRP) has been described (Brown & Spencer, 2013). During this period, the neonatal animal is reliant on maternal care to provide protection from outside impingements (Brown & Spencer, 2013). In human neonates, the SHRP period also appears to occur with the mother providing a buffer from outside stressors in order to ensure the development of a stable HPA axis by twelve months of age, (Gunnar & Donzella, 2002).

Research in pre-clinical animal models has shown that differential rearing, such as postnatal handling, maternal separation and early life stress result in behavioural changes evident in later life as anxiety, MDD, PTSD and increased levels of aggression (Clarke *et al.*, 1999; Jovanovic *et al.*, 2009; Lynch *et al.*, 2005; Miller *et al.*, 2002; Plotsky & Meaney, 1993; Pryce *et al.*, 2002; van den Berg *et al.*, 1999). Moreover,

these behavioural changes are associated with dysregulated HPA axis activation and immune system responses. Maternal deprivation is one of the most utilized and recognized forms of early life stress faced by infant animals (Raineki *et al.*, 2010). Attachment to a parental figure is considered to be a major requirement for the development of a healthy body as well as psychological health (Bowlby, 1897a, 1987b; Raineki *et al.*, 2010; Tang *et al.*, 2013). Moreover, early life stress has been found to alter the reactivity of the HPA axis which is dependent on the developmental age of the child and their genetic predisposition (Nader & Weems, 2011). Research, using an animal model of heel prick pain as experienced by neonates in an intensive care unit, showed there is an increased vulnerability to later stressors (Anand *et al.*, 1999). Thus, the pre-clinical protocol of disrupted maternal care has been used to assess the long term physiological impact of early life stress on the stress response system namely, the HPA axis (Levine, 2005). Furthermore, disruptions of the HPA axis have been associated with long-term behavioural and neurobiological changes (Clarke *et al.*, 1999; Glaser & Kiecolt-Glaser, 2005; Heim *et al.*, 2008; Lynch *et al.*, 2005; McEwen, 2007; Plotsky & Meaney, 1993; Pryce *et al.*, 2002; Raineki *et al.*, 2010; Rentesia *et al.*, 2013; Veenema, 2009).

Therefore, Bowlby's (1952) well established theoretical proposition, regarding the long term impact of insecure attachment on adult psychopathology, has increasingly been demonstrated by the biological evaluation of maternal deprivation research in animal models (Taylor, 2010; Jovanovic *et al.*, 2009; Veenema, 2009). Both the theory of attachment and the biological evaluation of maternal deprivation have enabled our

understanding of the impact of early life attachment difficulties in the development of later psychopathology and have demonstrated that altricial animals require good, consistent maternal care during early life for stable development of their stress modulation system (Fries *et al.*, 2005; Raineki *et al.*, 2010; Tang *et al.*, 2013).

1.2.2 Autonomic Nervous System

While the lasting impact of early life stress has been linked to changes in the HPA axis, it is important to note that the autonomic nervous system is also affected by stressful events (Hastings *et al.*, 2011; Pervanidou & Chrousos, 2012b). Synergistically acting with the HPA axis, the sympatho-adrenomedullary axis also responds to stressful situations to increase circulation levels of adrenaline for "fight-flight" responses such as increased heart rate, pupil dilation and increased bronchodilation to improve oxygenation. Centrally, the locus coeruleus in the brainstem regulates arousal and controls the secretion of peripheral nor-adrenalin (Pervanidou & Chrousos, 2012b). The sympathetic nervous system (SNS) responds almost immediately to a stressful situation by increasing adrenalin and nor-adrenalin secretion from the adrenal medulla and SNS neurons (Morris & Rao, 2013). Moreover, the sympathetic and parasympathetic nervous system are also known act in concert with the HPA axis (Hastings *et al.*, 2011; McEwen, 2006). However, sympathetic nervous system activation is relatively short lived and although we need to remain cognisant of the impact of the autonomic nervous system, a detailed review is beyond the scope of the present work

1.2.3 HPA axis

The HPA axis is the major stress response system that allows an organism the ability to adapt to the stressful situation over the longer term (Heim *et al.*, 2008; McEwen, 2003b, 2005). The HPA axis comprises both a central brain neuroendocrine aspect, namely the hypothalamus and the pituitary gland, and a peripheral somatic endocrine element, the adrenal gland. The HPA axis is highly conserved in vertebrates with the resultant hormonal cascade essential for daily responses to a stressful event (Levine, 2000). Cortisol (corticosterone in a rat model), the final hormone in the axis (See Figure 1), acts to enable the individual adapt to daily fluctuations and stressors. The regulatory control for response to a stressor is not as closely controlled as other homeostatic mechanisms and allows for a significant amount of change in stress hormone production when the organism encounters a stressor (Sterling, 2004).

Under excitatory control from the amygdala and inhibitory control from the hippocampus, stressors that are physical, psychological or environmental are sensed in the paraventricular nucleus of the hypothalamus, resulting in the release of the peptide corticotropin releasing hormone (CRH) into the portal blood stream of the hypophyseal stalk (Levine, 2000; 2005). CRH stimulates the release of production and secretion of the peptide adrenocorticotropic hormone (ACTH) from the anterior pituitary into the systemic blood stream. ACTH is then responsible for the secretion of the glucocorticoid cortisol, or corticosterone in the rodent, from the adrenal gland (Heim *et al.*, 2008; Levine, 2000, 2005). The HPA axis has a predictable 24 hour circadian rhythm with nadir secretion levels during sleep then gradually rising from approximately 04:00 in a

human diurnal pattern (see Figure 1) and reaching peak cortisol secretion at midday (for an extensive review see Kalsbeek *et al.*, 2012; Trifonova, *et al.*, 2013). After midday and as evening and darkness approach there is a steady decline in glucocorticoid production and an inverse increase in melatonin activity that signals the onset of sleep. Glucocorticoid release is associated with 180° phase difference between nocturnal species such as the rodent and diurnal species such as humans (de Boer & Van der Gugten, 1987; Kalsbeek *et. al*., 2012). Moreover, a negative feedback mechanism back to the hippocampus, hypothalamus and the pituitary maintains glucocorticoid levels to meet the fluctuating demand from the environment. In response to increasing daylight and behavioural activity the human cortisol awakening response (CAR), occurs within thirty minutes post awakening (Petrowski *et al.*, 2010). Awakening requires a surge of the glucocorticoid cortisol, thought to be important for preparing an individual for daytime activity and meeting the demands of the day (Akirav *et al.*, 2004; Clow *et al.*, 2010; Kalsbeek *et al.*, 2012).

Cortisol, a cholesterol-derived steroid hormone, binds to specific glucocorticoid receptors (GRs) found throughout the brain and in peripheral tissues (see Figure 4; Booij *et al.*, 2013; Herman & Spencer, 1998). Cytosolic glucocorticoid (GC) receptors are the key regulators of cortisol action in the brain (Herman & Spencer, 1998). Once cortisol has bound to the GC receptor it has the ability to affect the translation of a number of genes to produce the necessary proteins required (Herman & Spencer, 1998).

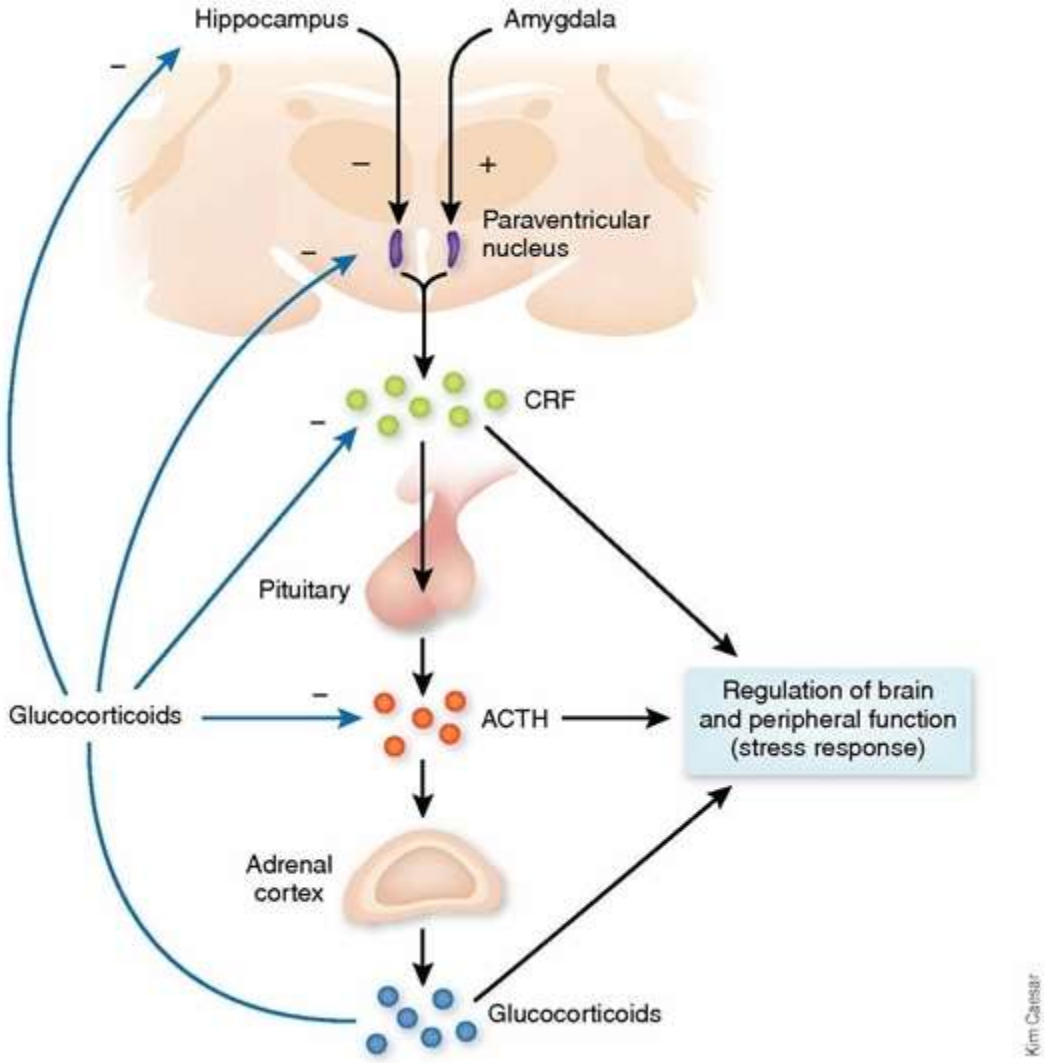


Figure 1. HPA axis stress response.

The HPA axis is under the excitatory control of the amygdala and inhibitory control of the hippocampus. In response to a stressor, the hypothalamic hormonal cascade commences at the paraventricular nucleus, releasing CRF, is transported to the corticotropic cells in the anterior pituitary, causing the release of ACTH into the blood stream. In turn, ACTH stimulates the adrenal cortex to synthesize and release the glucocorticoid cortisol (humans) or corticosterone (rodents). A feedback mechanism from the secreted glucocorticoid occurs back to the level of the hippocampus, hypothalamus and pituitary to dampen excess activation of the HPA axis. Reproduced with permission from Nature Neuroscience (Hyman, Nature Neuroscience, Vol 12, Issue 3, pages 241-243, 2009).

Although cortisol has a circadian rhythm, during a stressful situation, the adrenal cortex is able to adapt and secrete a bolus of cortisol into the peripheral blood stream to enable the organism to deal with the stress. Biological, psychological or environmental impingements from outside the HPA axis were considered by Hans Selye to be stressors that would result in an adaptive response by the organism (Fulford & Harbuz, 2005). Adaptation to a stressor is regulated with a pulsatile negative feedback mechanism back to the hypothalamus and the pituitary to maintain optimum cortisol levels. Ladd *et al.* (2004) provide evidence of reactive feedback changes in the HPA axis following early life stress with enhanced proactive feedback during the diurnal trough, but in response to an acute stressor there is a decreased reactive feedback resulting in prolonged responsiveness of the HPA following an acute stressor.

Following the experience of a stressor, there are a number of physiological responses, under the control of an intact amygdala, that occur to deal with the stressor (Sterling, 2004). The variability in stress hormone response has been termed allostasis by Sterling and Eyer (Sterling, 2004). Allostasis allows for "predictive regulation" (Sterling, 2004, p 26) in response to everyday normal stress responses. In Sterling's (2004) terms, the mechanism of allostasis allows for adaptation to meet the anticipated demands of daily living. The concept of an allostatic load has gained in popularity in stress research (McEwen, 1998a, 1998b; 2006; Katz *et al.*, 2012). A regulatory mechanism that allows for constant change is vulnerable to "wear and tear" especially if these challenges are severe, too early in developmental lifespan or persistent, resulting in an overloaded stress response unable to adjust to increased demand (Katz *et al.*, 2012). In terms of the

concept of allostatic load, McEwen (1998, 2006) documents four responses that may persist beyond the original stressful event namely, frequent increases in high levels of glucocorticoids due to continuing stressors in the individuals life, frequent increases in high levels of GC's in the absence of any real stressful event, prolonged increases of glucocorticoids after a stressful event and an inadequate response in the event of a new stressor (Figure 2). Katz *et al.* (2012) note that the measurement of allostatic load needs to be understood in terms of the initial physiological demand which primes the stress response for maladaptive secondary outcomes, or load, on the physiology of an individual. A growing understanding of the physiological load associated with early life trauma and the lasting impact that stress may have on the hypothalamic-pituitary-adrenal (HPA) axis and consequent cortisol secretion is changing the paradigm of psychiatric illness (Briere, 1992; Heim *et al.*, 2004, 2008).

Increasing evidence of a dysregulated HPA axis has been noted in adult patients who retrospectively report early life stress (Badanes *et al.*, 2011; Carpenter *et al.*, 2007; Danese & McEwen, 2012). In some patients diagnosed with a Major Depressive Disorder (DSM-5, APA 2013) potentiated HPA axis responses have been shown to develop in response to a subsequent stressor in later life (Heim *et al.*, 2000; Ladd *et al.*, 2004; Levine, 1957; Levine & Lewis 1959; Levine *et al.*, 1992; Veenema, 2009).

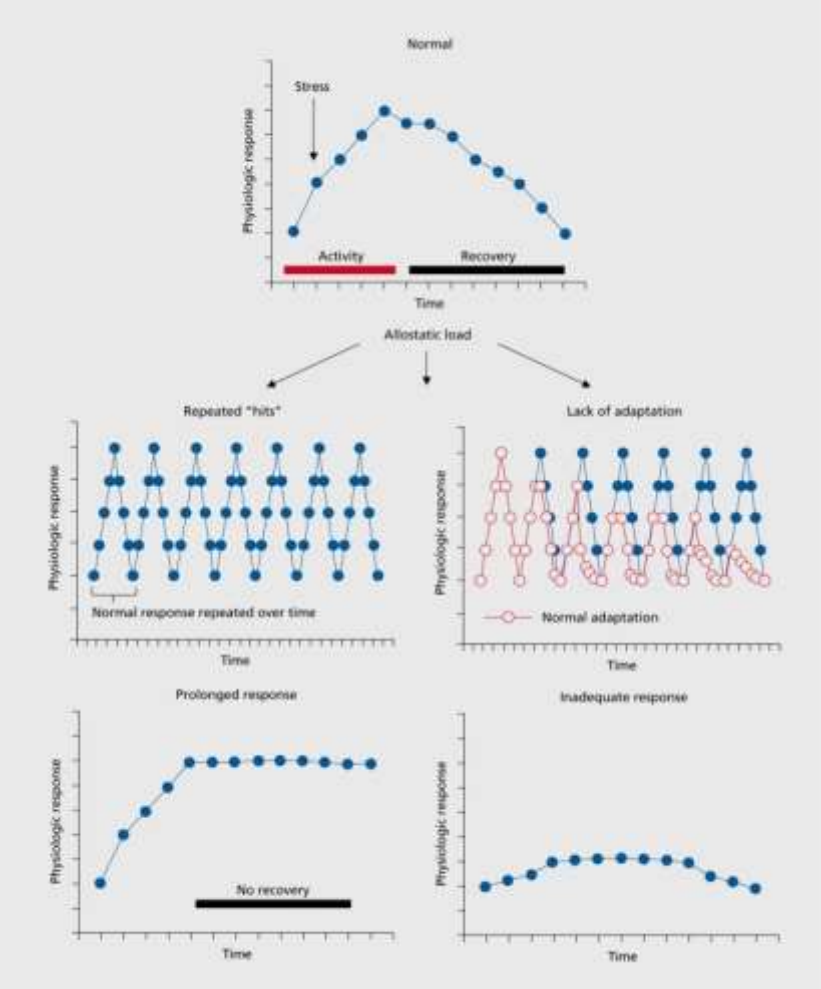


Figure 2. Allostatic load.

The normal stress response, with an increase in glucocorticoids at the time of the stressor followed by a return to baseline levels. In this figure, McEwen (1998a) details the normal response and four allostatic load conditions and responses that may occur following repeated stressful events viz. frequent unremitting stress, a lack of adaptation to a severe stress, continual high stress response or an inadequate response. "Reproduced with permission: **McEwen B.S. (1998a). Protective and damaging effects of stress mediators. New England Journal of Medicine, 338, 171-179. Copyright: Massachusetts Medical Society.**

Additionally, stress has been found to result in altered circulating levels of ACTH and CRF, with a dysregulated cortisol response to subsequent stressors (Carpenter *et al.*, 2007; Ladd *et al.*, 2004, Miller *et al.*, 2002). Administering an exogenous dose of

corticosterone stimulates disrupted attachment behaviours in an abusive maternal deprivation animal model of abusive attachment (Raineki *et al.*, 2010). Increased cortisol reactivity to a stressful task has also been shown in children who present with a disorganized attachment style (Bernard & Dozier, 2010). Moreover, higher attachment anxiety in adult women has been correlated with higher cortisol responses (Dewitte *et. al.*, 2010). A key factor in HPA axis dysregulation has been the impact of early life adversity. Primarily, the impact occurs in young children and animals as the stress response not fully developed and stabilized.

In contrast, in individuals without a psychiatric diagnosis, who retrospectively report early life stress show a decreased ACTH and cortisol response (Carpenter *et al.*, 2007) when exposed to the social laboratory stressor, the Trier Social Stress Test (TSST). The TSST requires individuals to present an idea to an antagonistic audience (Allen *et al.*, 2014). The test is known to induce stress in human research participants, (Allen *et al.*, 2014; Buchanan *et al.*, 2009). Similarly, in pre-clinical research (Beiko *et al.*, 2004) a marked reduction in plasma corticosterone levels following repeated exposure to the same stressor, indicative of a habituation to the stress, has been documented. Although a hyper-responsive hypothalamic-pituitary-adrenal (HPA) axis is one of the most common findings in neuroendocrine research and evident in large proportion of patients suffering from MDD, Heim *et al.* (2008) note that there is also the possibility that under severe early life stress there may be a down-regulation of the HPA axis and a decrease in the levels of circulating cortisol, as indicated by McEwen's (1998a) "inadequate" response (Figure 2). Seltzer *et al.* (2013) have recently shown an association between physical

abuse, in girls aged between 8 and 11.5 years of age, and a significant suppressive effect of salivary cortisol levels following an evening social stressor. Their data confirm the increasing evidence of a dampened HPA axis following significant early life stress when children are faced with another stressor (Fries *et al.*, 2005; Heim *et al.*, 2000). An increased allostatic load is considered to be responsible for an increase in vulnerability to later psychopathology in humans, particularly when faced with a future severe stressor such as rape or death of a parent (Goodman *et al.*, 2004; McEwen, 2000). Thus, altering adaptability of the HPA axis following major life stressors and the resultant physiological effects are considered to be aetiologically associated with long-term psychiatric pathologies (Boscarino, 2004; Bremner *et al.*, 2003; McEwen, 2000; Rinne *et al.*, 2003).

Importantly, following the early work by Levine (1957) on early life stress and the differential secretion of glucocorticoids there was a growing need to explain the evident behavioural changes in patients presenting with psychopathology. Behavioural descriptors are utilized significantly in the field of psychiatry due to the lack of biological biomarkers (APA, 2013; Berk, 2013; Bergink *et al.*, 2013; Insel, 2007). To address the link between behaviour and early life stress there has been an increasing amount of research into the neurobiological implications of HPA axis reactivity. In 2002, Scientific American ran an article entitled "Scars that won't heal: the neurobiology of child abuse" which highlighted the impact that early maltreatment has on brain development and neurotransmitter levels (Teicher, 2002). An extensive amount of evidence of neurobiological damage following stress has been related to HPA axis

dysregulation, specifically increased levels of cortisol (Heim *et al.*, 2004; McCory *et al.*, 2012). Early life stress is considered to prime the stress response system and to induce neurogenic changes in the developing brain of altricial species (Brown & Spencer, 2013).

Evaluation of HPA axis dysregulation

Evaluation of HPA axis dysregulation has been accomplished in a number of ways. Firstly, as noted, McEwen (2006) details the immediate response to a specific stressor. In the laboratory, one of the most common methods for evaluating a stress response is the Trier Social Stress Test (TSST) which requires individuals to present an idea to an antagonistic audience (Allen *et al.*, 2014). The test is known to induce stress in human research participants, (Allen *et al.*, 2014; Buchanan *et al.*, 2009). Similarly, in pre-clinical research, application of a specific stressor such as maternal deprivation or foot-shock is known to result in changes in corticosterone secretions (Beiko *et al.*, 2004, Levine, 1957; Veenema, 2009). Secondly, validated salivary cortisol measures have been used at specific time intervals during the day to evaluate cortisol secretions to generate a profile of HPA axis activity throughout the day (Linares *et al.*, 2008; Pervanidou *et al*., 2007). Closely associated with measuring daily salivary cortisol is the measurement of the cortisol awakening response (CAR) in a patient (Keeshin *et al*., 2013; Petrowski *et al.,* 2010). Thirdly, in order to evaluate 24 four hour glucocorticoid output, free cortisol can be measured in urine (Delahanty *et al*., 2000) or glucocorticoid metabolite output may be evaluated in faeces (Harper & Austad, 2000). Finally, to evaluate HPA axis regulation, a dexamethasone suppression test has also been administered. This test allows for the evaluation of the effect of exogenous

glucocorticoid dexamethasone on the feed-back mechanism (see Figure 1) of the HPA axis. Dexamethasone provides negative feedback to the hypothalamus and pituitary glands by binding to the glucocorticoid receptors to suppress the secretion of ACTH and CRH. Utilizing this test enables evaluation of the regulatory capability of both ACTH and CRH (Carvalho Fernando *et al.*, 2012).

Neurobiological effects of increases in glucocorticoids

The brain has synaptic plasticity which allows remodeling of the neuronal network under early life stressful conditions (McEwen, 2006; 2007). Moreover, the central nervous system is a heterogeneous organ, characterized by functionally different interconnected regions (Newman & Harris, 2009) which are a target for the stress hormone cortisol. Cortisol has a neurotoxic effect on some areas of the brain such as the hippocampus and a neurogenic effect on other areas such as the amygdala (McEwen, 2006, 2007; Raineki *et al.*, 2010). Moreover, the development of the various regions of the brain is not linear and occurs during different developmental stages (Wachs *et al.*, 2013). Thus, increased HPA axis reactivity after child maltreatment has been associated with neurobiological adaptations (Heim *et al.*, 2008). Ladd *et al.* (2004) found neurobiological changes in a pre-clinical study of rats maternally separated for 180 minutes from post-natal day (PND) 2-14 which demonstrated increased hippocampal mineralocorticoid receptor density with a decreased cortical and hippocampal glucocorticoid receptor density. In rodent models anxiety is associated with increased hippocampal mineralocorticoid activation, while decreased glucocorticoid functioning is associated with cognitive and spatial deficits (Ladd *et al.*, 2004). In patients diagnosed with BPD, Labudda *et al.* (2013) have reported an inverse relationship between the

severity of BPD symptoms and the volume of the left hippocampus. The hippocampus is required for regulatory control of the HPA axis (Buchanan *et al.,* 2009; Herman *et al.*, 2003). An intact hippocampus, rich with GC receptors, is also essential for learning and memory (Maletic *et al.,* 2007) and is also required for perception and evaluation of stressful events (Buchanan *et al.,* 2009). Patients with hippocampal damage do not exhibit an increase in cortisol during the Trier Social Stress Test. Interestingly, these patients showed increased levels of cortisol and sympathetic responses prior to the test but the test itself did not elicit an increase in cortisol (Buchanan *et al.*, 2009).

The hippocampus consists of a number of areas known as subfields namely, the dentate gyrus, CA-3 and subiculum each which have a large number of GC receptors that are a significant target area for cortisol and consequently, altering hippocampal function (Booij *et al.* 2013; Herman & Spencer 1998). Hippocampal neurones have been shown to exhibit shrinkage, structural remodelling and reduced neurogenesis following childhood maltreatment (Teicher *et al.,* 2012; Wachs *et al.,* 2013) and with increased levels of circulating glucocorticoids (McEwen, 2000; 2006; 2007).

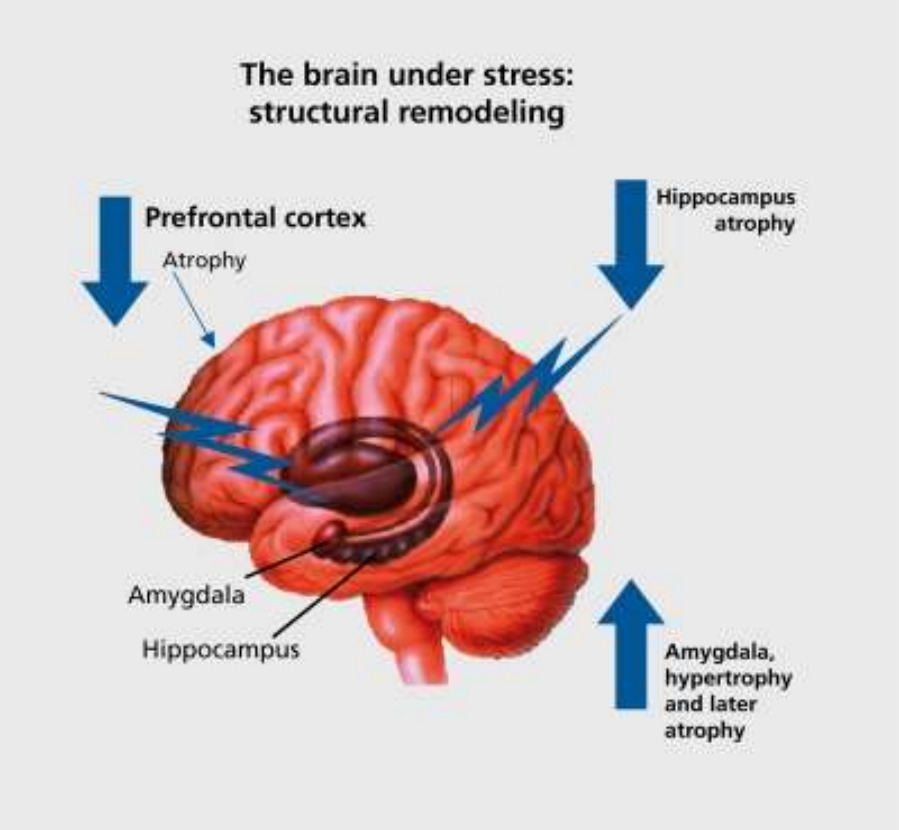


Figure 3. Remodelling of brain under stress

Remodelling of the cortico-limbic system occurs under stress. The cortico-limbic system consists of several brain regions that include the rostral anterior cingulate cortex, hippocampal formation, and basolateral amygdala. The anterior cingulate cortex has a central role in processing emotional experiences at the conscious level and selective attentional responses. Emotionally related learning is mediated through the interactions of the basolateral amygdala and hippocampal formation and motivational responses are processed through the dorsolateral prefrontal cortex. Reprinted with permission from an open-access article distributed under the terms of the Creative Commons Attribution License. McEwen B.S. (2006). Protective and damaging effects of stress mediators: central role of the brain. Dialogues of Clinical Neuroscience, 8, 367-381.

In contrast to the hippocampus, in the closely associated amygdala there is increased neurogenesis occurs under the influence of increased GC's. The amygdala is important for the evaluation of fearful situations (McEwen, 2007; Wachs *et al.*, 2013) and mediates aggressive impulses (Benes, 2010). Increased amygdala volumes are

considered to be a risk factor for the development of depression during adulthood (Lorenzetti *et al.*, 2009). In a pre-clinical model, following abusive maternal rearing, there was evidence of divergent neural circuits, increased neural activity in the basolateral complex and increased activity in the medial, cortical and central nuclei of the amygdala (Raineki *et al.*, 2010). Burghy *et al.* (2012) have noted that the consequent later life emotional reactivity following early life stress is associated with long-term changes in the neuronal connectivity between the amygdala and the ventromedial prefrontal cortex (vmPFC).

However, increased levels of glucocorticoids in the cortex result in neurodegeneration and shrinkage of dendrites following increased levels of stress (Burghy *et al.*, 2012; McEwen, 2006; Wachs *et al.*, 2013). The processing of emotional experiences, the regulation of affect and the determination of personality occurs primarily through neuronal connections from the medial pre-frontal cortex to the limbic system (hippocampus and amygdala) and anterior cingulate cortex (Benes, 2010; Burghy *et al.*, 2012; Gee *et al.*, 2013). The neural basis for self-regulation requires integrated connections between the orbito-frontal cortex and the ventral striatum (Wachs *et al.*, 2013). Importantly, changes in connectivity occur between these regions during development (Burghy *et al.*, 2012; Gee *et al.*, 2013). Adolescents parented in warm and caring environments have been shown to present superior intellectual abilities and higher temperamental control of emotion following positive parenting (Whittle *et al.*, 2013). In contrast, later life emotional reactivity is associated with neurobiological changes in the circuitry of the amygdala to the ventromedial prefrontal cortex following early life stress

(Burghy *et al.*, 2012). Increased GC's at specific developmental stages, namely infancy or adolescence, may cause shrinkage and degeneration of the neurones and dendrites in connecting these areas resulting in lifelong difficulties with immediate self-gratification and negative affect (Burghy *et al.*, 2012; Gee *et al.*, 2013; Wachs *et al.*, 2013).

Metabolic effects of glucocorticoids

As a stress hormone, one of the major effects of cortisol is to increase the release of glucose from tissues to ensure that the organism is able to engage in either fight or flight in a dangerous situation. However, once the threat has passed cortisol levels are expected to return to the normal baseline values (McEwen 1998a). In contrast, maintaining increased levels of vigilance, as would occur in early childhood maltreatment, may have the effect of disrupting this normal mechanism. Cortisol thus has a significant metabolic effect which enables the formation of new glucose from body fat and proteins (gluconeogenesis), the breakdown of fat (lipolysis) from certain areas of the body, moving the fat and storing it closer to the liver in the omentum and around the gastric organs. (Ibrahim, 2009). This visceral storage of fat is achieved due to the increased density of glucocorticoid receptors around the viscera (Ibrahim, 2009). Therefore, stress and the increase in circulating cortisol has been implicated in the development of obesity and will be covered in more detail in Section 3.1.

Epigenetic changes

While the role of genetics is evident in disorders such as autism and bipolar disorder it is the non-genetic disorders that form the greatest health burden in society (Hyman, 2009). Some psychological theorists have proposed that an underlying genetic vulnerability

results in later life psychopathology following early life stress (Goodman *et al.*, 2004; Gunderson & Lyons-Ruth, 2008). Gunderson and Lyons-Ruth (2008) emphatically state that BPD evolves in a child who is genetically predisposed to an interpersonally hypersensitive phenotype when exposed to environmental challenges.

Since the early days of psychological theory it was maintained that early life distress can affect health in later life (Bowlby, 1952). Hyman (2009) and Booij *et al.* (2013) suggest that epigenetic programming, due to altered HPA axis reactivity may provide a clearer understanding of the mechanisms by which early life stress primes an individual for later life pathology (Figure 4). Moreover, the understanding of the mechanisms by which early life stress alters the translation and transcription of genetic material may be essential for the development of treatments following early life stress (Hyman, 2009; Uher & Weaver, 2014). Although a full review of the epigenetic modifications is beyond the scope of this thesis, in sum, the genome of an individual provides for potential expression but it is the modified epigenome which identifies the specific aspects of DNA that are expressed (Booij *et al.*, 2013). By altering the methylation of DNA in a cell the glucocorticoid, cortisol, is a key regulatory mechanism for the translation of genetic material (Hayashi-Takagi *et al.*, 2013). Booij, *et al.* (2013) have recently hypothesized that significant methylation of DNA occurs during the perinatal period and is responsible for epigenetic changes over the long term to the HPA axis response to stressful events. Thus, mothering style has been implicated in epigenetic changes to methylation of the glucocorticoid receptor gene which is thought to alter stress responses in later life (Murgatroyd, 2014; Sapolsky, 2004; Weaver *et al.*, 2004).

Furthermore, early life stress has been found to alter DNA methylation and the consequent reprogramming of both the HPA axis and the serotonin neurotransmitter system and may account for long term psychiatric outcomes (Booij *et al.,* 2013; Hayashi-Takagi *et al.*, 2013; Uher & Weaver, 2014). Investigations into the severity of bipolar mood disorder have found that increased traumatic events namely, sexual, physical and emotional, in early life result in a greater percentage of methylation of the promoter region on the glucocorticoid receptor gene labeled NR3C1 (Perroud *et al.* (2014). These authors hypothesize that increased severity of bipolar mood disorder may be due to the degree of DNA methylation in the NR3C1 promoter and the consequent epigenetic modifications of glucocorticoid receptor resulting in changes to the HPA axis hormonal cascade (Perroud *et al.*, 2014).

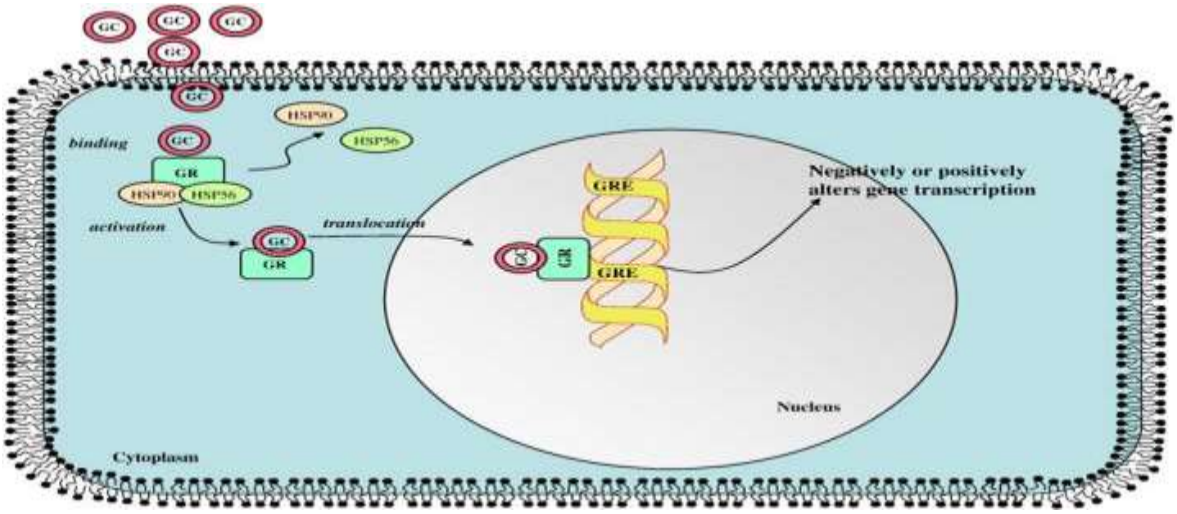


Figure 4. Glucocorticoid receptor (GR)

Endogenous glucocorticoids (GC) act as glucocortiocoid receptor (GR) ligands. Once the GR is activated, the GC's translocate from the cytoplasm to the nucleus and thereby gene transcription. Gene transcription occurs by binding to hormone response elements on DNA or by interacting with other transcription factors. Reproduced with permission from Juruena, M. (2013). Early-life stress and HPA axis trigger recurrent adulthood depression. Epilepsy & Behavior, http://dx.doi.org/10.1016/j.yebeh.2013.10.020

In translational research from a rat model of early life stress and epigenetic programming of the hippocampal glucocorticoid receptor, McGowan *et al.* (2009) examined the hippocampus of suicide victims and found that childhood maltreatment was associated with changes to hippocampal glucocorticoid receptors. McGowan *et al's*. (2009) results add support for previous work which indicated that increased HPA reactivity is associated with decreased glucocorticoid receptors in the cortico-limbic system, while a decrease in HPA activity is associated with an overexpression of glucocorticoid receptors. Korosi *et al.* (2012) have developed a three-way interaction of genes, environment and development model for determination of vulnerability to psychopathology and Perroud *et al.* (2014) have demonstrated the applicability of the model with bipolar mood disorder patients (Uher & Weaver, 2014). Thus, early adverse events can modulate the expression of molecules involved in cellular plasticity within the hippocampus that thereby contribute to permanent alterations in brain structure and function, which might ultimately lead to an increased vulnerability to psychiatric diseases (Korosi *et al.*, 2012; Perroud *et al.*, 2014).

A further possible biomarker of stress, the length of the telomere, has emerged in the past decade. Telomeres, DNA nucleobases, form a repetitive sequence of guanine, adenine, thymine, and cytosine and are represented using the letters G, A, T, and C. The TTAGGG sequence at the end of linear chromosomes provides replication stability for the genetic material (Shalev *et al.*, 2012). In the assessment of three different forms of abuse, namely, exposure to domestic violence, physical maltreatment and bullying, Shalev *et al.* (2012) found that telomeres can be eroded from a very early age in the face

of cumulative violence. Children exposed to two or more forms of violence were more likely to have increased erosion of telomeres (Shalev *et al.*, 2012). However, as the authors note, their study raises a question regarding the physiological processes that may be considered causative for telomere damage. Inflammation and oxidative stress are considered to be significant factors in damaging telomere length. Adverse rearing and stressful events in animal models have shown altered programming of both the HPA axis and the immune system during critical developmental periods (Anisman *et al.*, 2002; Korosi *et al.*, 2012; O' Conner *et al.*, 2003). Thus the link between childhood stress, inflammation and future biological and physiological perturbations is enhanced (Shalev *et al.*, 2012).

1.2.4 Immune system dysregulation

Cortisol is a potent anti-inflammatory agent with significant bi-directional effects with the immune system (Figure 5; Blalock *et al.*, 1985; Trifonova *et al.*, 2013). The HPA axis has been shown to exert control over the immune system during infectious and inflammatory processes (Blalock *et al.*, 1985; Borghetti *et al.*, 2009; McEwen *et al.*, 1997). However, the extreme complexity of the immune system has, until recently, rendered it relatively impenetrable to research. In recent years, investigations into the relationship between the endocrine system, principally the hypothalamic-pituitary-adrenal (HPA) axis, and the immune system have become an important area of stress research as there is now considerable evidence that some psychiatric disorders may occur as a result of inadequate inflammatory control by the glucocorticoids (Raison & Miller, 2013a). Of note is that the milieu of endocrine and immune system responses to

stress may result in nuances that are not easily distinguished by only one biological marker, such as cortisol.

Therefore it is feasible that it is not HPA axis reactivity per se, but rather the relationship between cumulative stressors, the endocrine and the immune system that results in an allostatic load (Faturi *et al.*, 2010). Early work by Maes *et al.* (1993) on the relationship between the HPA axis and the immune system using the dexamethasone suppression test, administration of exogenous glucocorticoid to evaluate the production of ACTH from the pituitary gland (see Figure 1), demonstrated that 33% of the variation in cortisol production could be attributed to the IL-6 cytokine production by peripheral blood mononuclear cells (PBMC's).

The inflammatory response is known to be induced by infective organisms. However, in recent years, it has become evident that acute and chronic stressors also induce an inflammatory response (Black, 2003). Importantly, an acute phase response is the initial immune response of the body to infective organisms, tissue damage and stress (Black, 2003). An inflammatory group of immune system messengers, the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, have been found to be increased in response to infection (Hamblin, 1993).

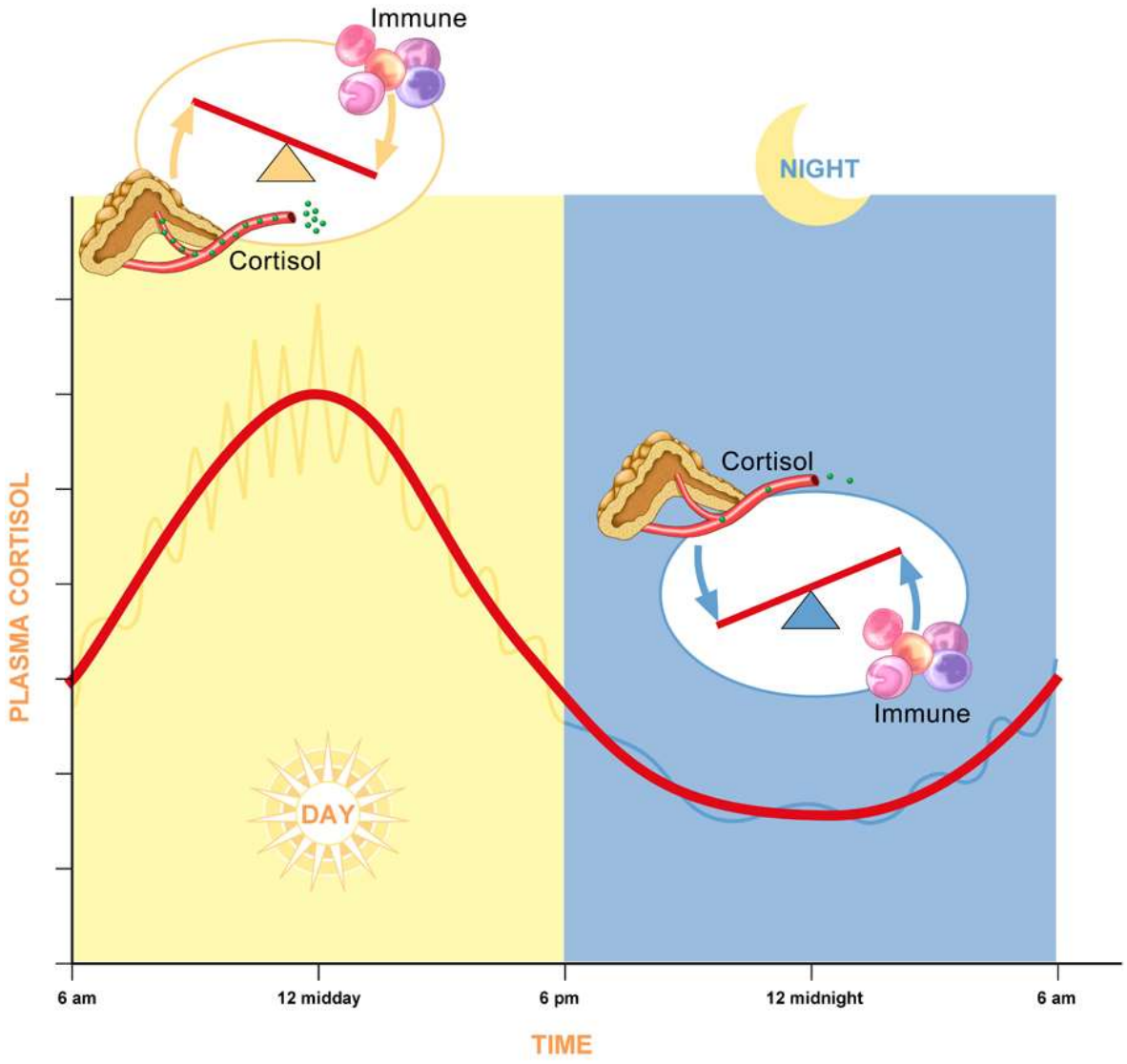


Figure 5. Circadian rhythms

Graphic illustration of the circadian relationship between cortisol and the immune system. Bi-directional modulation between cortisol and the immune system changes during a twenty-four hour period. Increased cortisol during the day allows the individual to handle daily stressors (Blalock *et al.*, 1985; Trifonova *et al.*, 2013; Van Houdenhove *et al.*, 2009). Conversely decreased cortisol secretion at night allows the immune system to repair areas of the body that have been damaged. Copyright: Denise Muller (2014), School of Physiology, Health Sciences, University of the Witwatersrand.

Ground breaking work on sickness behaviours associated with infective processes led to the realisation that some symptoms of depression resemble those that occur following

infections (Dantzer & Kelley, 2007; Maes *et al.* 1993). Sickness behaviour, a well-established response to infection of fever, malaise, altered appetite, pain and fatigue has been noted to resemble depressive symptoms in both humans and animals (Anisman *et al.*, 2005; .Dantzer & Kelley, 2007).

Thus, there has been a growing realization that immune system dysregulation may be a contributor to the development of the lasting sequelae from early life stress and HPA axis perturbations. Moreover, chronic stress has been shown to dysregulate immune system responses (Dhabhar, 2009). The complex relationship that exists between the HPA axis and the immune system allows for the regulation of each system (see Figure 5) by the other and has been extensively investigated in the past two decades (Maes *et al.*, 1993; Raison *et al.*, 2006). Moreover, it has become increasingly evident that inflammatory processes may play a role in the development of psychiatric disorders such as depression (Maes *et al.*, 1993; Mills *et al.*, 2013; Raison *et al.*, 2006; Raison & Miller, 2013a, 2013b; Shelton & Miller, 2011) and in somatic disorders such as cardiovascular disease and metabolic disorders such as obesity (Hyland *et al.*, 2012; Shelton & Miller, 2010). Importantly, Harrison *et al.* (2009) have shown that inflammatory processes following a vaccination for typhoid results in mood changes with associated increased activity in areas such as subgenual anterior cingulate cortex (sACC) and reduced connections from the sACC to the amygdala and the medial prefrontal cortex.

Early childhood stress and increased risk of inflammatory processes has been shown in a longitudinal study in which maltreated children had a significant risk of increased C-

Reactive Protein (CRP) twenty years later (Danese *et al.*, 2007). The authors note that more than 10% of low grade inflammation in a population may be attributed to early childhood stress (Danese *et al.*, 2007). The pro-inflammatory cytokines interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor – alpha (TNF-α) have also been found to be raised following severe stress in humans and animals (Johnson *et al.*, 2003). Cytokines have also been investigated with respect to the development of depression in cancer patients receiving immunomodulatary therapy with interleukin -2 (IL-2) and interferon-alpha (IFN-α; Anisman *et al.*, 2005; Raison *et al.*, 2006). These new developments of cancer care by cytokine recombinant treatments have resulted in the development of depression that was greater than that which occurred in patients receiving conventional cancer treatments (Raison *et al.*, 2006). A striking resemblance between sickness behaviour and depression was noted when the immune system messenger molecules, the cytokines IL-1, interferon-gamma (IFN-g) and IL-2, were cloned to treat patients with cancer (Anisman *et al.*, 2005; Raison *et al.*, 2006). The finding that symptoms, that resembled sickness behaviour, were induced in these patients led to the knowledge that there is communication between the peripheral circulation and the central nervous system (Dantzer & Kelley, 2007).

A number of studies have shown that the immune system and the HPA axis respond to a second stressor with increased levels of circulating pro-inflammatory cytokines and a potentiated HPA axis response (Johnson *et al.*, 2003; Pugh *et al.*, 2001). However, as O'Conner *et al.* (2003) have found, early life stress may prime immune system cytokine response to a later social stress and lead to an enhanced or dysregulated physiological

response. Cytokines communicate with other cells such as macrophages and the dendritic cells of the central nervous system to control inflammation. However, dysregulation in different cytokines may lead to a number of symptoms such as fatigue, sleep disturbances, anhedonia and changes in eating behaviour (Anisman *et al.*, 2005). Some of the key cytokines that have been investigated with respect to depression include IL-6, IL-2 and IL-α (Anisman *et al.*, 2005; Raison *et al.*, 2006). Moreover, aberrations in immune system activation in the cytokines IL-1, TNF-α, and interferon gamma (IF-γ) have also been noted to influence neuronal function (Hayley *et al.*, 2005, Raison & Miller 2013a).

Upregulation of the enzyme indoleamine 2,3 dioxygenase (IDO) by immune system cytokines has been noted to result in changes to neurotransmitters serotonin and dopamine (Anisman *et al.*, 2005; Raison *et al.*, 2010). During infective processes, cytokines have been found to upregulate IDO to sequester tryptophan, required for serotonin production, away from neurotransmitter metabolism to the L-kynurenine pathway. L-kynurenine is further metabolized to quinolinic acid and kynurenic acid. In infective states, this pathway provides increased protective anti-inflammatory regulation of the immune system (Dantzer *et al.*, 2008; Raison *et al.*, 2010). Behaviourally, activation of the L-kynurenine pathway is evidenced by sickness behaviour. However, as Dantzer *et al.* (2010) note, there is as yet little evidence which cytokines are important in the development of later life disorders following early life stress.

As noted previously, it is well established that maternal deprivation results in alterations to the HPA axis. However, there has been contradictory evidence regarding the long-

term implications of the HPA axis response. Attenuation in plasma cortisol levels and enhanced cytokine response such as IL-6 may have either deleterious or beneficial effects dependent on the time of day when the perturbations occur (Figure 5).

Interleukin-6 (IL-6)

IL-6, a pleiotropic cytokine, is considered to be a significant biomarker in human psychopathology (Kahl *et al.*, 2006; Pervanidou, 2008). IL-6 dysregulation has also been noted in patients with depression (Anisman *et al.*, 2002, 2005), in children and adolescents with Post Traumatic Stress Disorder (Pervanidou, 2008) and in adult medical patients with co-morbid depression (Anisman, 2009; Raison *et al.*, 2006; Raison & Miller, 2011). In particular, as IL-6 is an immune system acute phase protein, the role of IL-6 has been investigated following early life stress (Black, 2003) and following a psychosocial stressor in healthy adults (Izawa *et al.*, 2013b). In research on the impact of early child abuse, daily stress in adulthood has been associated with a twofold increase in circulating plasma IL-6 concentrations compared to adults who have not experienced early childhood abuse (Gouin *et al.*, 2012). Immune system dysregulation, as measured by alterations in diurnal concentrations of IL-6, have also been noted in patients who present with BPD and who retrospectively report early life adversity (Kahl *et al.*, 2006).

IL-6, a pro-inflammatory cytokine, is synthesised by a large number of immunologically active cells and plays a key role in regulation of the immune system (Hamblin, 1993; Maes *et al.*, 1993). The biological functions of IL-6 include production of the innate immune system molecule, the acute phase protein CRP in the liver, co-stimulation of the

adaptive immune system T-cells, B cell differentiation, and differentiation of cytotoxic lymphocytes (Hamblin, 1993). Musselman *et al.* (2001) note that in patients with depression there is an increased level of circulating IL-6. Moreover, as previously described, using an ex-vivo experiment with peripheral blood mononuclear cells following the dexamethasone suppression test, Maes *et al.* (1993) showed that IL-6 secretion by the PBMC's was significantly positively correlated with increased cortisol secretion. Miller and Cole (2012) have found evidence to suggest that inflammatory markers such as IL-6 are increased in certain sub-populations of adolescents at risk for depression. Importantly, they note that depression in adolescents who had experienced childhood adversity was associated with an increase in IL-6 six months prior to the onset of the disorder (Miller & Cole, 2012). Dysregulation of IL-6 has also been shown by Musselman *et al.* (2001) to be significantly higher in patients with cancer and associated untreated depressive symptoms. Furthermore, peripheral increases in circulating IL-6, following typhoid vaccination was associated with the mood changes noted by Harrison *et al.* (2009) and as previously noted, are also associated with changes in activity subgenual anterior cingulate cortex (sACC) with reduced neuronal connections from the sACC to the amygdala and the medial prefrontal cortex.

In pre-clinical animal studies, the most common model for evaluating later life immune system dysregulation is maternal deprivation (Anisman *et al.*, 2005; Connor *et al.*, 2005). Increased levels of IL-6 have been found in the colonic secretions of maternally deprived rats following a later life stressor (O'Mahony *et al.*, 2009; O'Malley *et al.*, 2011). IL-6 has also been shown to be associated with increased nociceptive pain

responses in adulthood in a rat model of erratic care (Alvarez *et al.*, 2013). Nociceptive pain responses in female rats following maternal deprivation are also associated with increased levels of IL-6 in the hippocampus (Burke *et al.*, 2013). Moreover, in a pain study of muscle hyperalgesia, using a limited bedding model in the early postnatal period rather than maternal deprivation, Alverez *et al.* (2013) have shown that IL-6 levels are increased. Although changes to the pro-inflammatory cytokine milieu, specifically IL-6, are evident in later stages of development, the possibility exists that other immune system cells may provide more promising data at an early developmental age (Dantzer & Kelley, 2007; Dhabhar *et al.*, 2012; Khanfer *et al.*, 2010; Miller *et al.*, 2009).

White Blood Cells

Glucocorticoids are recognised for their significant anti-inflammatory actions (Coutinho & Chapman, 2011). The glucocorticoid cortisol has been shown to have specific effects on different cell populations allowing for the differentiation of specific white blood (WBC) cells, the leukocytes (Coutinho & Chapman, 2011). Acute stress has been shown to enhance immune responses and a leukocytosis whereas chronic stress has been found to result in a suppression of white cell production, leukopenia (Shi *et al*., 2003). Importantly, the glucocorticoids also allow immune system cells to be redistributed to areas of the body that require surveillance (Dhabhar & McEwen, 2007; Dhabhar *et al.*, 2012). In a pre-clinical model of acute stress Dhabhar *et al.* (2012) have shown that redistribution of WBC's is a critical survival mechanism when an individual is infected by microorganisms. Acute stress has been shown to result in a mobilization of WBC's into the circulation while a decrease in plasma WBC's may reflect the redistribution of

the cells into specific body compartments (Dhabhar *et al.*, 2012). In previously stressed mice, increased movement of monocytes into the brain from the spleen has been shown to be associated with recurrence of anxiety (Wohleb *et al.*, 2014). In clinical populations, increased circulating white blood cells have also been associated with obesity (Benson *et al.*, 2008; Phelan *et al.*, 2011). However, Raison and Miller (2013a, 2013b) caution, that slight changes in inflammatory markers that occur during episodes of stress may not be detectable during the early stages of chronic stress and the cumulative effect of slight changes may account for significant impacts on neuroendocrinology in later development. The possibility thus exists that the inflammatory process following severe early life stress may also be implicated in the aetiology of psychiatric disorders, specifically BPD.

Thus, there are clinical implications of early life stress, HPA axis and immune system dysregulation in adult patients who present with psychiatric and somatic disorders. In developing countries, although infectious agents remain a significant cause of death, the increasing prevalence of mental health disorders and the development of diseases of lifestyle are of concern (WHO, World Health Organization, 2008, 2009). Mental illness prevalence rates are estimated at approximately 25% worldwide at an estimated cost of $2500 billion in 2010 (Kinderman *et al.*, 2013). The health burden of psychiatric disorders has been documented by Kessler *et al.* (2009) following the WHO World Mental Health (WMH) surveys on the global burden of mental disorders. Similarly, there is an increase in the health burden of lifestyle associated diseases such as obesity, metabolic syndrome and cardiovascular disease (Schmidt *et al.*, 2008). The following

section comprises (1) a review of the mental health disorders associated with early life stress and co-morbidly associated with BPD and (2) a review of the somatic disorders co-morbidly evident in patients with BPD.

2. Psychiatric Disorders

Traumatic childhood histories and a dysregulated HPA axis have been shown to be more likely in adults experiencing depression (Heim *et al.*, 2008), PTSD (Delahanty *et al.*, 2004; Jonkman *et al.*, 2013) and BPD (Carvalho Fernando *et al.*, 2012; Rinne *et al.*, 2003). In an extensive meta-analytic study of sexual abuse associated with psychiatric diagnoses, Chen *et al.* (2010) note that sexual abuse survivors comprise 13-26% of first world primary care practices. Although the WHO consortium (Collins *et al.*, 2011) have recognised an worldwide increase in the mental health burden, there appears to be a lack of focus on the possible aetiological impact of early life stress, particularly in developing countries. In contrast, our understanding of early life stress in the aetiology of some of the psychiatric disorders has undergone a major paradigm shift (Heim *et al.*, 2008).

In the last 20 years, the relationship between early life stress, physiological stress responses, the immune system and a number of psychiatric disorders has been extensively explored (Heim *et al.*, 2008). Thus the existence of psychiatric disorders in later life has been noted to be associated with prior stress and the consequent allostatic load that the individual has experienced (Wachs *et al.*, 2013). Moreover, disorders identified in the DSM-5 such as depression, anxiety and post-traumatic stress disorder (Delahanty *et al.*, 2004; Goodman et. al. 2004; Maughan and Rutter, 1997; Rinne *et al.*, 2003) and the personality disorder BPD (Kahl *et al.*, 2006) have been retrospectively

linked to adverse early life experiences. According to Dhabhar (2009) the only reliable evidence for the role of early life stress and the impact of future stressors is by understanding the effect that stress has on the biological system of an individual. The impact of stress on both the endocrine and immune systems has a cascading impact on downstream peptides, neurotransmitters and translation of genetic material which may be beneficial or harmful (Dhabhar & McEwen, 2007; Dhabhar, 2009). In the following section, I will review the current field of research into specific psychiatric disorders, co-morbidly associated with BPD, associated with early life stress and previously investigated with respect to both HPA axis and/or immune system dysregulation.

2.1. Major Depressive Disorder

Depression is frequently noted to be co-morbidly associated with patients presenting with BPD (Kahl *et al.*, 2006). MDD is diagnosable when a patient meets five or more specific behavioural criteria as delineated in the DSM-5 (APA, 2013). Depression has been noted to be the third leading contributor to the global burden of health, affecting approximately 65 million people worldwide (Collins *et al.*, 2011). The DSM-5 criteria for depression include a loss of enjoyment of daily life, depressed mood as well as changes in eating and sleeping patterns for a period of at least two weeks (APA, 2013). However, while the DSM-5 has tried to document all the possibilities that may exist it is evident that the DSM-5 diagnostic criteria remain a ”subjective list of behavioural adjectives”, comprised of a great deal of variability with no definitive pathophysiological variable (Berk, 2013, pp 128.). Furthermore, the apparent heterogeneity of depression and the current lack of an established aetiology (Hayley *et al.*, 2005; Heim *et al.*, 2004) may have an impact on the treatment that patients receive.

A meta-analytic study of sexual abuse and psychiatric disorders shows that a significant relationship exists between a history of sexual abuse and the development of depression (Chen *et al.*, 2010). Moreover, a significant amount of research has documented the role that glucocorticoids have in altering neurobiochemistry (Irwin & Miller, 2007; Miller *et al.,* 1999; Hayley *et al.,* 2005). The HPA axis has been extensively investigated with respect to the development of depression and self-reported early life stress (Carvalho Fernando *et al.*, 2012; Heim *et al.*, 2000; 2004).

Over the last two decades it has become increasingly evident that both the HPA axis, specifically glucocorticoid secretion and receptor function, play a key role in the development of depression (Maletic *et al.*, 2007). In depression there is evidence of an imbalance of cortisol secretion, altered GR and MR receptors and sensitivity to the glucocorticoids (Booij *et al.,* 2013). Using a dexamethasone suppression test Carvalho Fernando *et al.* (2012) found moderate correlations between psychopathology and early life stress adding support for McGowan *et al's* (2009) assertion that early life stress alters central glucocorticoid receptor functioning. In patients who retrospectively reported early life stress, higher cortisol levels were found both before and after administration of an oral dose of dexamethasone 0.5mg (Carvalho Fernando *et al.*, 2012). Higher cortisol levels were positively correlated with dissociative symptoms and the severity of depression (Carvalho Fernando *et al.*, 2012). While a return to a normal HPA axis response to the Trier Social Stress Test in depressive patients is considered to be evidence of remission (Lange *et al.*, 2013).

Furthermore, there is also increasing evidence that the immune system may also be dysregulated in depression. As noted, Maes *et al.* (1993) demonstrated that IL-6 secretion from patients diagnosed with melancholic depression was positively correlated with increased post dexamethasone cortisol levels. In adolescents previously exposed to early life adversity, increased levels of the biomarkers CRP and IL-6 preceded a depressive episode six months later (Miller & Cole, 2012). Moreover, a link has also been documented between depression, the HPA axis and the pro-inflammatory cytokines, Il-1β, TNF-α, IL-6 and interferon-γ (Hayley *et al.*, 2005; Irwin & Miller, 2007; Lang & Borgwardt, 2013; Maes *et al.*, 1993). The role of the cytokines in depression has been highlighted in a number of studies that have isolated elevated plasma cytokines and other inflammatory markers such as CRP during a depressive episode (Hayley *et al.*, 2005; Kahl *et al.*, 2006; Miller *et al.*, 1999). IL-6 has been noted to be increased in patients with depression and in patients being treated for cancer who present with higher than normally expected depressive symptoms (Musselman *et al.*, 2001).

2.2 Post Traumatic Stress Disorder.

Severe trauma is known to induce post-traumatic stress symptoms in victims. The diagnostic criteria for PTSD, described in the DSM-5 (APA, 2013), for children older than six, adolescents and adults is clear. Following exposure to a severe traumatic event, PTSD diagnostic criteria include the subsequent development of characteristic symptoms, such as frequent re-experiencing of the event with associated behavioural and emotional responses in which some individuals may become emotionally volatile. However, emotional responses in other patients may present as anhedonic and dysphoric

(APA 2013). Although the diagnosis of PTSD in the earlier version of the Diagnostic and Statistical Manual of Mental Disorders 3rd edition (DSM-III R), had only been established with respect to experiences of war, natural catastrophes and accidents (Kaplan & Sadock, 1988), increasing focus on victims of interpersonal trauma such as torture, rape and assault has led to the realization that the symptoms of PTSD are also evident in these patients (Briere, 1992). The long-term effects of sexual abuse of children has been shown to produce post-traumatic symptoms in some victims and these victims are now being considered as exhibiting the symptoms of a chronic post-traumatic stress disorder (Rinne *et al.*, 2002). Some researchers argue that the labile emotionality evident in BPD patients is a chronic form of PTSD, following childhood abuse which has altered the personality development of the child (Briere, 1992).

Early research into PTSD amongst Vietnam veterans concentrated extensively on the effects of cortisol and HPA axis variability with respect to the behavioural responses of veterans seeking treatment (Mason *et al.,* 2002). Their research into PTSD yielded contradictory results in inpatient veterans over a period of 90 days indicating that some personality coping mechanisms might alter cortisol responses to a stressor (Mason *et al.*, 2002). Thus the confounding variables which indicate a greater severity of symptoms such as poorly controlled anger, persecutory ideation and shame as measured on the psychiatric Minnesota Multiphasic Personality Inventory (MMPI-2) are associated with significant differences in cortisol secretion under an acute stress. Therefore, the researchers concluded that research into PTSD and HPA axis lability should include a personality inventory and should be conducted longitudinally to ascertain which

personalities are affected more severely than others with either low or increased cortisol secretion (Mason *et al.,* 2002). In addition, they suggest that the role of co-morbid psychiatric disorders such as depression and anxiety, which are known to show elevated cortisol concentrations, should be evaluated to ascertain whether they are causative or the result of the alterations in the HPA axis feedback mechanisms in patients diagnosed with PTSD (Delahanty & Nugent, 2006; Mason *et al.*, 2002). Moreover, investigations into the relationship between PTSD and urinary cortisol secretion have shown an increase in urinary cortisol concentrations associated with increased duration and severity of PTSD symptoms in children who have been maltreated (De Bellis *et. al*., 1999). McGowan (2013) highlights the fact that early life stress is a risk factor for the development of PTSD following a later life trauma due to the early epigenetic changes to the glucocorticoid receptor in the corticolimbic system. In addition, IL-6 dysregulation has also been noted in children and adolescents with Post Traumatic Stress Disorder (Pervanidou, 2008). Increased levels of IL-6 may result in neuro-inflammation and the consequent neurobiological changed associated with PTSD.

The debate thus continues regarding the applicability of the current diagnostic criteria for PTSD. Herman (2012) has argued for the inclusion of a classification of complex posttraumatic stress disorder (CPTSD) to be considered for inclusion into DSM-5 (Herman, 2012). However, Resick *et al.* (2012) maintain that there is insufficient research data to consider a new category. Moreover, the lack of diagnostic criteria for children younger than six years of age and the inadequate account for the associated impact of developmental stages that occur concurrently with the experience of trauma

should be considered (Ford *et al.*, 2013). It is possible that the impact of acute or chronic trauma occurring during specific developmental ages in a child's life may account for the development of later life personality disorders, specifically BPD.

2.3 Borderline Personality Disorder

BPD is prevalent in 1-6% of the general population and due to the significant distress that patients experience has been found to be economically costly (Belsky *et al.*, 2012). BPD patients are remarkably difficult to characterize and are frequently intransigent to treatment. The complexity of personality involvement in BPD has resulted in fewer physiological investigations than in depressive, anxiety and post-traumatic stress disorder (Wingenfeld *et al.*, 2010). As noted, attachment difficulties are one of the most significant element of the BPD diagnosis resulting in significant impairment in personality functioning. Thus, BPD has been described as a set of personality traits and behaviour that are considered not to conform to the norms established by the individual's culture (APA, 2013). An important factor in the diagnosis of BPD is the significant distress that the patient experiences in daily functioning. and in interpersonal relationships (APA, 2013). Significant debate continues regarding the role of trauma in the development of BPD disorder (Goodman *et. al*., 2004). The diathesis stress model maintains that some individuals are more likely to develop BPD, regardless of traumatic exposure, due to a familial inherited risk (Belsky *et al.*, 2012; Gunderson & Lyons-Ruth, 2008). In contrast Herman *et al.* (1989) maintain that traumatic early life experiences alter the physiology and neurobiology of the individual subsequently leading to the development of BPD.

Abuse histories are common in patients diagnosed with BPD with eighty-one percent of BPD patients retrospectively reporting physical abuse, sexual abuse and the witness of serious domestic violence (Herman *et al.,* 1989). In the late 1980's it became evident that even moderate endocrine malfunction may have an impact on personality development (Briere, 1992). Moreover, as noted, there are indications that some of the diagnostic features of the DSM-5 BPD characterization resemble chronic post-traumatic stress symptoms (Briere, 1992). The manifestations of anxiety, lack of concentration, eating disorders, bizarre flashbacks in the form of nightmares, self-harm behaviour and volatile mood are more reminiscent of PTSD rather than personality features (Briere, 1992; Rinne *et al.,* 2003).

Thus, although BPD has traditionally been theorised to be psychological in nature, specifically with respect to the personality aspects (Kernberg, 1993), research over the last three decades has demonstrated that altered cortisol concentrations, HPA axis activation and immune system perturbations following stressful events are evident in BPD patients (Lee *et al.,* 2012; Kahl *et al.,* 2006). Neurobiological changes in the hippocampus have also been reported (Goodman *et al.,* 2013). Moreover, treatment with a selective serotonin reuptake inhibitor such as fluvoxamine is associated with a significant reduction in ACTH and cortisol response to a dexamethasone suppression test (Rinne *et al.,* 2003). There is now considerable opinion that severe and early stress may result in an increase in plasma cortisol prior to the development of the symptoms such as BPD, psychoses or depression (Rinne *et al.,* 2003). However, Lee *et al.,* (2012) found that adult patients with personality disorders and childhood histories of adversity

have a blunted cortisol and ACTH response to the dexamethasone challenge. Consequently, activation of the HPA axis needs to be the focus of research and treatment for this disorder and not only the central nervous system serotonergic neurotransmitter system (Rinne *et al.,* 2003). Research has thus demonstrated that symptoms following severe stress, previously considered to be psychogenic, are significantly related to the neuroendocrine and immune system physiological functioning of the BPD patient (Kahl *et al.*, 2006; Rinne *et al.,* 2002; 2003; Teicher, 2002). However, BPD is also associated with impairments to physical health (El-Gabalawy *et al.,* 2010). Moreover, comorbid BPD symptoms and somatic health conditions result in a poor quality of life with increased morbidity and mortality (El-Gabalawy *et al.,* 2010; Powers & Oltmanns, 2012).

3. Somatic Disorders

In addition to early life stress resulting in evidence of allostatic load in psychiatric symptoms, allostatic load may also be evident in the lifestyle disorders such as cardiovascular disease, obesity and metabolic syndrome (Wachs *et al.*, 2013). Interestingly, the WHO (2009), have not considered early life stress to be a risk factor for non-communicable diseases. The World Health Organisation (WHO, 2009) lists ten risk factors as leading causes of death. Of note is that regardless of income category, each list includes four cardiometabolic risk factors, namely hypertension, increased blood glucose, obesity and high cholesterol (WHO, 2009). In recent years cardiometabolic disease has increasingly been investigated with respect to early life stress and dysregulation of the HPA axis and immune system.

As noted in the section 3, psychiatric diagnoses continue to be made on behavioural evidence. The reliance on behavioural descriptors has resulted in the under diagnosis of somatic stress symptoms in distressed children and the frequent response that "children are resilient". In research with adult patients it is becoming increasingly evident that early life stress may induce long term somatic disorders that may not be behaviourally evident when a child first presents for medical care (Paras *et al.*, 2009). Therefore the focus in this section is on the increasing evidence which links early life stress and BPD with the disorders of lifestyle, obesity, diabetes mellitus Type II, metabolic syndrome and cardiovascular disorder.

3.1 Obesity and Metabolic Syndrome.

Stored fat, particularly in the visceral compartment of the body, is required for availability during times of famine and stress. Evolutionary biology suggests that the visceral mechanism of fat storage, close to the liver and with increased numbers of glucocorticoid receptors, is beneficial for survival (Ibrahim, 2009; Wajchenberg, 2000). However, in times of plenty, as found in most western economies, the deposition of increased visceral fat has resulted in the development of the so-called "diseases of lifestyle". Poor diet and sedentary lifestyles have been implicated in the development of metabolic syndrome with prevalence estimates between 13-35% in developing countries and high income economies such as the USA and the UK (Dunkley *et al.*, 2012). Interestingly in obesity prevalence data (Dunkley *et al.*, 2012) and in Wajchenberg's(2000) extensive review, there is no mention of the role that stress may play in the development of obesity. In contrast, in a recent study in adult patients diagnosed with eating disorders, Castellini *et al's*. (2012) findings on a complex inter-

relationship between eating disorders, childhood sexual and physical abuse and HPA axis functioning suggest that eating disorders may be more common in abused children. A history of rape has also been significantly associated with eating disorders (Chen *et al.*, 2010; Fischer *et al.*, 2010). Furthermore, attachment difficulties, which Tasca *et al.* (2013) characterized as affect dysregulation and interpersonal sensitivity, have recently been shown by to be associated with eating disorders. Additionally, these authors note that disordered eating should be assessed as possible evidence of distress. Abuse in childhood and adolescence are also predictive of increased visceral fat, a precursor of Type II diabetes mellitus, and strongly associated with obesity in adult women with both MDD and BPD (Kahl *et al.*, 2005; Rich-Edwards *et al.*, 2010).

Moreover, in the last decade there has been increased focus on the relationship between stress, psychiatric disease and the development of obesity and metabolic syndrome (Després, 2006; Lang & Borgwardt, 2013; McIntyre *et al.*, 2007; Pervanidou & Chrousos, 2012a; Shelton & Miller, 2010). Metabolic syndrome is a constellation of medical disorders which include hypertension, dyslipidaemia, raised cholesterol, fasting hyperinsulinaemia and blood glucose (Després, 2006; Dunkley *et al.*, 2012). The relationship between metabolic dysregulation associated with depression is becoming increasingly evident with some authors noting that depression is a metabolic disorder (Lang & Borgwardt, 2013; McIntyre *et al.*, 2007). Therefore, the question regarding the developmental impact of early life stress and HPA alterations with respect to the aetiology of obesity is an important one. Patterson and Abizaid (2013) argue that stress may be the aetiological factor that is driving the current obesity epidemic, firstly due to

the increased level of stress found in modern societies and possibly also due to the sleep deprivation that individuals face when needing to travel further and further to a work-place. Fatigue, personality style and increased levels of aggression are associated with increased feeding behaviours, hyperinsulinaemia and increased abdominal obesity (Raikkonen *et al.*, 1996). Moreover, in BPD patients, increases in the level of visceral fat and obesity have been noted (Frankenburg & Zanarini, 2006; Kahl *et al.*, 2005; Roepke *et al.*, 2010). There is mounting evidence of the role of the adipocyte as an endocrine and immune system organ (Galic *et al.*, 2010; Harwood, 2012). Adipokines, adipose tissue messengers, are involved in energy homeostasis, acting both centrally and peripherally to regulate processes such as eating, energy expenditure and carbohydrate and lipid metabolism (see Figure 6; Harwood, 2012; Shelton & Miller, 2010). Immunologically, the secretory functions of adipose tissue have been found to be significantly associated with macrophages, resulting in a low level of chronic inflammation (Galic *et al.*, 2010; Shelton & Miller, 2010).

Thus, central adiposity, evidenced by an increase in waist circumference, is a marked feature of metabolic syndrome and is associated with increased CRP levels (Miller et al., 2013). Soldiers diagnosed with PTSD have a higher incidence of central obesity, hypertension, dyslipidaemia and Type II diabetes mellitus (Levine et al., 2013). Levine et al. (2013) suggest that PTSD management of returning combat veterans include full evaluations for cardiometabolic syndrome. Moreover, the veterans with PTSD also present with immune system dysregulation with disorders such as increased susceptibility to infections and gastric ulcers (Levine et al., 2013). The stress of daily

living requires an increase in HPA axis activation, with associated increased levels of cortisol and therefore, due to the glucocorticoid effect on energy homeostasis, an increased need to supply energy in the form of carbohydrate intensive foods (Patterson & Abizaid, 2013).

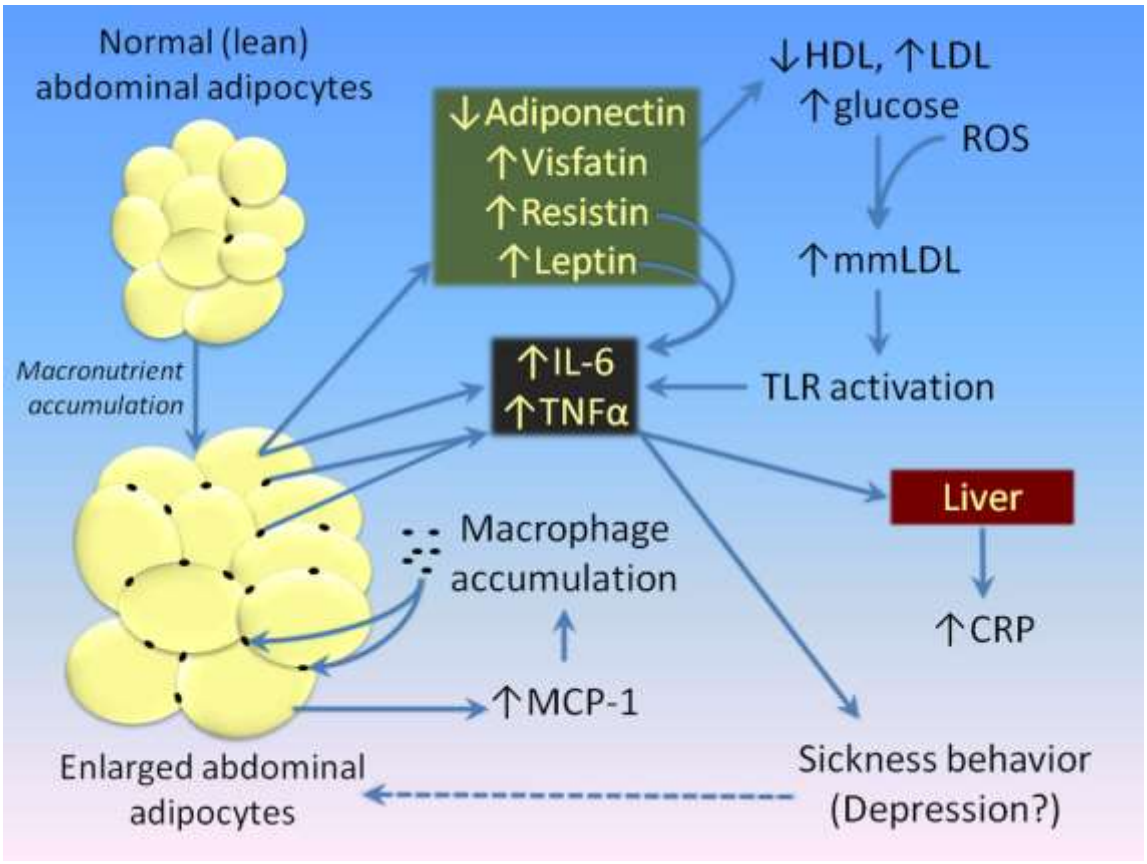


Figure 6. Adiposity and inflammation.

High caloric intake in the diet leads to increased accumulations of lipids in adipocytes. Increased lipid content results in an increased release of MCP-1 (CCL2), a chemoattractant that increases the infiltration of macrophages into adipose tissue. Both adipocytes and macrophages release inflammatory mediators such as IL-6 and TNFα into the peripheral circulation. This, coupled with adverse effects on adipocytokines and related molecules such as leptin, resistin, visfatin, and adiponectin is thought to mediate the relationship between accumulation of adipose tissue and conditions such as dyslipidemias and diabetes. Low density lipoprotein cholesterol is oxidized to form minimally-modified LDL's (mmLDL), which also can activate toll-like receptors, further stimulating the production of cytokines. Increases in peripheral cytokines may, then, lead to depression. However, depression may also enhance the accumulation of body fat, further aggravating the process of adipose-induced inflammation. Reproduced

with permission from Shelton & Miller (2010). Eating ourselves to death (and despair): the contribution of adiposity and inflammation to depression. Progress in Neurobiology, 91, 275-299.

Increasing evidence in the current obesity epidemic suggests that HPA axis and immune system dysregulations and early increases in body mass may be important in detecting allostatic load in response to early life stressors (Jahng, 2011). Vogelzangs *et al.* (2007) have found that depressed mood and metabolic syndrome are characterized by an increase in urinary cortisol levels in older adults. Counter-intuitively, an increase in body mass has also been associated with a blunted cortisol response to stress in pre-school children who experienced chronic stress in a poor environment (Miller *et al.*, 2013). In an animal model, early life stressors have been shown to interact with environmental factors such as readily available palatable fast foods and portend a long-term impact on metabolic function (Bernardi *et al.*, 2013). Moreover, early life stressors have been shown to upregulate hypothalamic neurones, via CRF, called the orexins which increase appetitive behaviour (Chen *et al.*, 2014).

Gillman and Ludwig (2013) note that the prevention of obesity needs to start during infancy, as early life stress is associated with dysregulations in hormonal patterns that may be irreversible. Thus, the obesity epidemic and disorders of lifestyle such as the cardiovascular disorders have highlighted the growing relationship between early and later life stressors, HPA axis dysregulation, inflammatory processes and obesity. Additionally, plasma IL-6 is increased in obese patients and appears to be related to an infiltration of macrophages to areas of fat deposits (Galic *et al.*, 2012). However, the aetiology of obesity and the concomitant metabolic disruptions is as yet unclear. Metabolic syndrome has been found to be associated with a chronic low grade

inflammatory state (Miller *et al.*, 2013) and an increased risk of cardiovascular disorders (Dunkley *et al.*, 2012).

3.2 Cardiovascular disorders

Cardiovascular disease (CVD) is ranked in the top two causes of death by the WHO (2008) in low, medium and high income countries. In the United States of America, CVD accounts for approximately one-third of all deaths per annum (Logan & Barksdale, 2008). Patients diagnosed with BPD, have a known risk factor, increased intima-media thickness, in the common carotid artery and consequently may have an increased risk for the development of CVD (Greggersen *et al.*, 2011). The growing rate of cardiovascular disease has been linked to an increased level of societal stress and concomitant allostatic load that is experienced by patients (Logan & Barksdale, 2008). A link between childhood sexual abuse and myocardial infarction in adult men, but not women, has been noted with an odds ratio of 2.96 (Fuller-Thompson *et al.*, 2012). However, the growing obesity epidemic and specifically visceral obesity is associated with increased risk of cardiovascular disease (Dunkley *et al.*, 2012. Research into the relationship of early life stress, HPA axis dysregulation and immune interaction in the development of cardiovascular disease is in its infancy (Patterson & Abizaid, 2013). Early life stress, HPA axis dysregulation and depression have been associated with an increased risk of cardiovascular disease (Phillips *et al.*, 2013; Vogelzangs *et al.*, 2007).

Patients presenting a blunted cortisol response when exposed to a physical or a psychological stressor are noted to be more at risk for the development of CVD (Lu et *al.*, 2013; Nijm *et al.*, 2007). Phillips *et al.* (2013) have highlighted the long term

impact on health of an attenuated cortisol response to a stressor seen in some patients who present with cardiovascular disease. Moreover, HPA dysregulation in patients recovering from a myocardial infarct has been demonstrated by an increased bedtime cortisol response, matched with an increased 24 hour cortisol output (Nijm *et al.* (2007). Moreover, the blunted cortisol reactivity to the stressor was associated with disinhibited inflammatory responses as measured by an increased CRP (Nijm *et al.*, 2007). Thus, counter-intuitively, it has become evident that a blunted reactivity to stress, as a result of prior life stressors such as childhood maltreatment, may also lead to negative cardiovascular outcomes (Phillips *et al.*, 2013).

Dysregulated HPA axis responses, with impaired cortisol responses and associated increase in inflammatory processes, have also been implicated in negative outcomes in patients with cardiovascular disease (Hueston & Deak, 2013; Nijm *et al.*, 2007). Atherosclerotic lesions have been associated with increased inflammatory process and with significant psychological stressors (Black, & Garbutt, 2002). Stress can engender an acute phase response with increases in macrophages, immune system cytokines and inflammatory acute phase proteins such as CRP (Black & Garbutt, 2002; Nijm *et al.*, 2007). Poole *et al.* (2013) have recently shown that patients who rate higher on the Beck Depression Inventory prior to coronary bypass surgery had higher CRP levels and had a higher odds ratio of a longer stay in hospital post the bypass surgery.

4. Moderating factors found to ameliorate long-term disease severity

The evidence presented for disruption of the HPA axis and the immune system may lead to the conclusion that all neonates and children exposed to trauma will develop either a

psychiatric disorder or a somatic condition. However, there is a disparity between exposure to traumatic events and the development of long-term sequelae (Bartolomucci, 2005). Thus, there appear to be ameliorating factors that prevent long time disease. These factors include, good-enough maternal care (Tang *et al.*, 2013; Winnicott, 1987), genetic resilience (Pally, 2002); socio-economic status (Lupien *et al.*, 2000) and group support (Pyter *et al.*, 2014).

4.1 Maternal care

Good maternal care is considered to be a significant ameliorating factor in preventing attenuated cortisol responses during stressful events (Lyons *et al.* 2010; Tang *et al.*, 2013). Support for Winnicott's (1986) thesis of good-enough maternal care has recently been demonstrated by Whittle *et al.* (2013). Positive maternal care is predictive of decreased volume in the right amygdala (associated with fear responses), and accelerated cortical thinning in the left and right orbitofrontal cortices. Furthermore, Whittle *et al's* (2013) results provide support for Belsky's (2001) thesis that higher quality child care has implications for cognitive and academic achievement in adolescence. Positive parenting and higher quality childcare also predict less externalizing behaviour in adolescence (Vandell *et al.*, 2010; Whittle *et al.*, 2013). A critical factor for positive cognitive, behavioral, and psychological development is supportive, warm and caring, parenting early in life (Whittle *et al.*, 2013).

4.2 Genetic resilience

The diathesis stress model maintains that some individuals may be more at risk for the development of psychiatric and somatic disorder due to inherited genetic material

(Belsky *et al.*, 2012; Gunderson & Lyons-Ruth, 2008). The development of a disorder such as BPD has been linked to genetic predisposition due to the evidence that BPD occurs in families and is more common in monozygotic twins (Pally, 2002). However, as abuse tends to occur in families early life stressors may be conveyed to the younger members of a family with environmental factors facilitating or ameliorating existing genetic vulnerabilities (Pally, 2002). Thus, although genetic vulnerabilities should be considered, it would appear that there is an increased focus on the impact that early life stress, both prenatal and postnatal, has on glucocorticoid levels and consequently on epigenetically altering DNA translation (see Figure 4).

4.3 Socio-economic status

Lower socio-economic status (SES) has been associated with increased exposure to stress and consequently an increase in allostatic load (Karlamangla *et al.*, 2013; McEwen & Gianaros, 2010; Wachs *et al.*, 2013). Lower SES groups have been shown to have a greater risk for the development of chronic disorders and are evident in the earliest years of life (Karlamangla *et al.*, 2013). Stress in a family, particularly the mother's SES status and mood has an impact on the wellbeing of the child (Lupien *et al.*, 2000). From Lupien *et al's* (2000) study it would appear that there is a gradual increase in stress hormone reactivity in children from lower SES backgrounds. However, these authors also showed that increased cortisol levels in all children are correlated with mother's depression score.

4.4 Group support

Social isolation is considered to be a long-term sub-optimal stressor that may affect inflammatory processes in a diverse range of disorders (Hermes *et al.*, 2005). Social isolation and loneliness may have significant effects on future health (Pyter *et al.*, 2014). In humans, self-reported social isolation following a severe stressor such as rape or childhood abuse is a frequently reported occurrence without adequate means of evaluating the levels of support an individual experiences (Hostinar & Gunnar, 2013). A rat model in which social isolation is induced post a severe stressor may provide a physiological approximation of the isolation that is felt by children following a severe trauma. Recent research by Pyter *et al.* (2014) demonstrates that social isolation impairs wound healing. Importantly, Hostinar and Gunnar (2013) note that the evaluation of the protective mechanisms associated with social support lags behind the evaluation of stress responses.

5. Thesis Aim - Evaluation of biomarkers at specified early developmental stages

In adult patients with both psychiatric and somatic disorders there is a link between retrospective reporting of early life stress, associated with altered circulating cortisol and the cytokine IL-6. The relevant peripheral biological markers cortisol and IL-6 in adult patients has not been demonstrated in children when they first present to clinicians following the experience of a severe early life stressor. Child abuse is recognised as a significant risk factor for the development of long term physiological dysfunction (Paris, 1993). Moreover, clinicians such as Herman (2012) and van der Kolk (2005) have proposed that a category be established for developmental traumatic disorders.

However, the lack of good biologic markers associated with developmental stages makes it difficult to develop a nuanced diagnostic algorithm for establishing risk.

Importantly, given the extensive research detailed above with respect to the impact of stress on neurobiology, it would seem that another study investigating the impact of early life stress on neurobiology would be redundant in a developing country. However, at present a large part of the neurobiological research is not clinically applicable in a third world setting as convenient and cost effective access to medical advances such as fMRI scans is not possible. Thus, there is a growing need to find accessible and easily monitored biomarkers that offer diagnostic, prognostic and possible treatment options to medical professionals who treat distressed children. As suggested by McDade *et al.* (2013), a developmental approach to the study of depression and inflammation that will allow for earlier treatment of children who are stressed, i.e. during a prodromal stage of development, the more likely the possibility of preventing lasting neurobiological insults. My research parallels the search for a biological signature that allows for early diagnosis and treatment in other disorders such as schizophrenia, depression and anxiety (Schwarz & Bahn, 2008; Schwarz *et al.*, 2012).

Furthermore, notwithstanding the extensive documentation that has been generated in the 21st Century regarding early life stress, biological perturbations and future psychiatric and somatic conditions, we still do not have effective diagnostic tools for the evaluation of distress in young children. In research with patients presenting with PTSD with or without co-morbid BPD the only significant difference between two groups was

that abuse that occurred at an earlier developmental age was more likely to be associated with a diagnosis of BPD (Heffernan & Cloitre, 2000). This thesis is based on the growing realisation that we need effective diagnostic, prognostic and therapeutic tools to ameliorate the impact of early life stress. Therefore, although the main aim of this thesis is to investigate whether physiological perturbations are evident in children who present to a sexual abuse clinic, work with sexually abused children is complicated by a number of confounding variables, specifically maternal care.

Therefore, the major questions that require further clarification are:

- Is dysregulation of the HPA axis evident in young children who are exposed to the traumatic stress of abuse?
- Is there evidence that inadequate maternal care, during the neonatal stage of development, has an impact of HPA and immune function and consequently on body mass?
- Is there evidence of HPA axis and immune dysregulation occurring during subsequent stress responses during adolescence?

Investigations into particular stressors and the development age when the stress is experienced is becoming a significant factor in our understanding of disease progression (D'Andrea *et. al*., 2012). Moreover, there may also be a spectrum of severity and phenotypes that exist following early life stress. Thus, I have hypothesised that a developmental approach is required to establish the physiological response to stress evident at a specific stage. Additionally, few studies have firmly established the link to the immune system, specifically with respect to the relevant cytokines released when a

child is traumatised. With accumulating evidence of cytokine changes and the associated disorders in adult patients (Fagundes *et al.*, 2013; Glaser & Kiecolt-Glaser, 2005; O'Brien *et al.*, 2004), it is becoming increasingly important to diagnose endocrine and immune system dysregulation as early as possible to understand and then prevent the longer term effects.

5.1 Investigating developmental stress responses

The present research must be regarded as a preliminary investigation into possibilities for biological identification and the establishment of relevant biomarkers prior to the development of the sequelae of abuse. As Wachs *et al.* (2013) note, there are indications that preventative and therapeutic interventions need to commence as soon as possible after a severe stressor to maintain integrity in immune and neuroendocrine systems. This work should be seen as an early reflection of a new way in which to conceptualise the symptomatology of abuse for children who have experienced early life stress. The approach taken here is a broad one. While it has been recognised that there is a need in the field of development and child abuse for detailed distinctions, the present work attempts to provide a broader unified view of the impact of trauma on biological functioning with particular focus on the search for a biomarkers and the identification of allostatic loads that may aid in early diagnosis and consequently early treatment. Once the biological links between of abuse and physiology are clearly delineated and documented, it will be easier to draw attention to the specific differences in sequelae for victims of abuse. A view which addresses the lasting impact of abuse, in the context of developmental theory and clinical process, can enrich the fields of research and therapy

leading to a greater understanding of the client. As part of my Declaration, the contributions of each author to each of the studies submitted for publication are outlined below. Chapters 2 and 3 document the physiological impact of early life stress in the form of sexual abuse in children. The findings of decreased circulating plasma cortisol in children who have experienced other stressors, such as the loss of maternal care, highlight the need to develop a pre-clinical animal model to investigate perturbations in the identified biological markers cortisol and IL-6 at the specified developmental ages. Thus, Chapter 4 and 5 utilize a rat model to investigate maternal deprivation followed by a second later life stressor. The structure of the following chapters is as follows:

Chapter 2 - Cortisol and IL-6 Responses to Stress in Female Children Presenting at a Sexual Abuse Clinic.

The effect of childhood rape and incest has been considered as causative in the development of BPD (Briere, 1992). However, the studies are inconsistent as not all children who are raped develop the disorder. Additionally, human studies are retrospective and rely on the patient's recall of the events and the impact that it may have had on them. Retrospective reports from patients with BPD implicate both early childhood maternal deprivation and subsequent early life stressors such as sexual abuse (Goodman *et al.*, 2004). Thus, I investigated the relationship of cortisol and IL-6 in a small pilot study group of female children who presented to the forensic Teddy Bear Clinic with suspected sexual abuse, which was subsequently confirmed.

This pilot study confirmed the relationship between morning cortisol and IL-6 plasma concentrations. In particular, we found that children who were resident in a children's home presented with an apparent blunted cortisol response but with an enhanced level of IL-6 following the severe stress of the forensic examination. Importantly this study confirmed Bowlby's (1952) theoretical position regarding the impact of maternal deprivation and institutionalization. However, at the time of the pilot study, we were unable to find an applicable control group. Although we had ethics approval to recruit patients from both the Learning Disability clinic and the Epilepsy clinic, there were too many confounding variables. Thus, having secured a control group from a bone density study, we repeated the research at the Teddy Bear Clinic (see Chapter 3).

Chapter 3: Disparate plasma cortisol concentrations in sexually abused female children from Johannesburg, South Africa.

In chapter 3, the follow up study of the initial pilot research is presented. Following the forensic examination of an additional group of children from the sexual abuse clinic we confirmed differential plasma cortisol and IL-6 responses to the stress of the forensic examination. However, unlike our preliminary research when the children who were resident in a children's home had attenuated cortisol responses, the children with attenuated cortisol responses in this research were those children who had experienced significant other severe stressors, such as the death of their mother or abuse within the family home. Furthermore, in our non-abused control group of children exposed to a novelty stressor, a bone density scan and blood sampling, we identified a homogenous cortisol concentration with very little variance.

Therefore, this study highlighted the need to develop a systematic investigation into the impact of early life stress, at the neonatal stage of developmental. One of the key confounding variables in the study of human children is whether there is physiological evidence of maternal deprivation. Consequently, we designed a pre-clinical animal developmental study to investigate the impact of maternal deprivation on specific developmental stages, namely at the end of the neonatal period and following a severe stressor in the juvenile stage.

Chapter 4 - Variation of maternal deprivation protocol reveals increased body mass and visceral fat mass in female Sprague Dawley rat pups.

In chapter 4, the first pre-clinical project designed to establish a model for evaluating the long-term impact of early neonatal stress on both HPA axis activation and the immune response is detailed. The hypothesis for this study was that early deprivation is identifiable at an early developmental stage and is evident in the biomarkers corticosterone and IL-6. Early identification of disruptions in both the HPA axis and the immune system following early life adversity may prove useful in providing a targetable objective biological marker for early diagnosis and treatment (First & Zimmermann, 2006; Schwarz & Bahn, 2008; Schwarz *et al.*, 2012). Thus, I established that there are significant differences in glucocorticoid corticosterone responses to the stress of weaning in the neonatal development stage in our rat model. Moreover, these differences confirm the stress responses noted in the children who presented with earlier stressors such as maternal loss or other stressors in the home. Consequently there was a

need to identify, using a pre-clinical animal model, whether these differences are still evident following a further stressor in the juvenile (adolescent) stage of development.

Chapter 5 - Increased visceral obesity in juvenile female Sprague-Dawley rats following cumulative life stressors: Implications for future research.

In Chapter 5, data from the project with juvenile rats exposed to maternal deprivation and subsequently exposed to second stressor, namely foot-shock is presented. However, a lack of significant differences for plasma corticosterone and IL-6 but a significant difference in body mass emerged in the juvenile animals that had been maternally deprived, resulting in the generation of further hypotheses which were undertaken in a subsequent study. In Phase 2 of the project we also investigated whether there were differences in faecal corticosterone concentrations, WBC counts, body mass and visceral fat mass over the three foot-shock events. Following phase 2 we noted that there are significant differences in body mass, visceral fat content and white blood cell counts. Importantly, the ongoing work in our Stresslab is focused on the implications of early life stress during the juvenile phase of development.

CHAPTER 2

CORTISOL AND IL-6 RESPONSES TO STRESS IN FEMALE CHILDREN PRESENTING AT A SEXUAL ABUSE CLINIC.

Muller, D.M., Errington, S., Szabo, C.P., Pitts, N., & Jacklin, L.

Accepted for publication by the Journal of Child and Adolescent Trauma

Abstract

Since adults with histories of sexual abuse as children experience both dysregulation of cortisol and increased inflammatory markers, we hypothesized that plasma cortisol dysregulation and increased plasma IL-6 would be detectable at first presentation of girls between the ages of 6-12 years to a sexual abuse clinic. Following the stressful forensic examination the eleven patients recruited to the study had significantly different cortisol concentrations ($p<0.0075$), depending on whether they resided with family (309 ± 101 nmol/l) or in a children's home (157 ± 38 nmol/l). IL-6 was detected in all patients residing in a children's home, with plasma cortisol and IL-6 being inversely correlated ($r = -0.8875$). Our study demonstrates an association between decreased cortisol secretion, inflammation and place of residence in sexually abused girls.

Introduction

Adult and adolescent patients who present clinically with borderline personality disorder (BPD), major depressive disorder (MDD) and post-traumatic stress disorder (PTSD) frequently report retrospective associations with adverse early life experiences, specifically sexual abuse, in their formative years (Gratz *et al.*, 2008; Schoedl, *et al.*, 2010; van der Kolk, Roth *et al.*, 2005). Furthermore, following early life sexual abuse, patients with BPD and MDD (Kahl *et al.*, 2006), depression (Heim *et al.*, 2008) and women with PTSD (Gill *et al.*, 2008; Jovanovic *et al.*, 2009) have been found to present clinically with dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis. However, the impact of stress on cortisol concentration is contradictory in adult psychiatric patients since patients have been found to present with either a hypocortisolaemia, hypercortisolaemia or an apparently normal response (Badanes *et al.*, 2011; De Bellis *et al.*, 2011; Dhabhar, 2009; Heim *et al.*, 2000). Furthermore, characterizing the stress response using only cortisol as the key identified biological stress marker may limit efforts to provide interventions to patients who have been sexually abused. Thus, in addition to cortisol, the immune system inflammatory marker, interleukin-6 (IL-6), has been shown to significantly increase in adult patients diagnosed with BPD and comorbid major depression (Kahl *et al.*, 2006), depression (Pace *et al.*, 2006; Raison, Capuron, & Miller, 2006; Raison, & Miller, 2011) and PTSD (Gill *et al.*, 2008).

Severe stress may alter physiological balance and adaptability in the individual and result in an imbalance, an "allostatic load", on both the endocrine and immune systems

(McEwen, 2000, p174). Therefore, an imbalance of immune system cytokines, following severe psychological trauma, may be an early marker of long-term allostatic load (McEwen, 2000) and future immunopathology (Dhabhar, 2009) in young patients. Although retrospective reporting of abuse is linked to dysregulation of the HPA axis and IL-6 (Kahl *et al.*, 2006), the immunological impact of HPA axis activation on the immune system has not been fully investigated in children who present at child abuse clinics. Pervanidou *et al.* (2007) have shown that elevated morning plasma IL-6 concentrations are evident in children and adolescents who experience PTSD following a motor vehicle accident. However, at present, little information exists regarding the relationship between the immune system and the endocrine system in sexually abused distressed children. In a recent study on the lasting impact of sexual abuse in children in South Africa, Mathews *et al.* (2013), note that a significant number of children have sustained levels of PTSD six months post disclosure of sexual abuse. Furthermore, adult psychiatric patients who present with BPD and MDD retrospectively report early life sexual abuse (Chen *et al.*, 2010; Gratz *et al.*, 2008; Kahl *et al.*, 2006). Additionally, these patients have also been noted to present with evidence of cortisol and immune system dysregulation (Heim *et al.*, 2008; Kahl *et al.*, 2006; Rinne *et al.*, 2003) which is frequently resolved using antidepressant (Loman & Gunnar, 2010; Rinne *et al.*, 2003).

Thus, in addition to understanding behavioral and emotional adaptations to complex psychological trauma (Jonkman *et al.*, 2013; Mathews *et al.*, 2013) it is important to know the extent of endocrine and immune system dysregulation in young children. Early life stress may have a priming effect on the HPA axis resulting in a lifelong

imbalance in stress modulation (Juruena, 2013). Additionally, early life stress and HPA axis dysfunction has been associated with decreased hippocampal volumes (Labudda *et al.*, 2013). In female patients, greater severity of BPD is associated with decreased volumes in the left hippocampus and the parahippocampal area (Labudda *et al.*, 2013). Thus, there is a growing need to determine possible biomarkers at first presentation of early life stress related to sexual abuse (Ayer *et al.*, 2013).

Our prospective, proof of concept study in female children who have experienced sexual abuse is documented here. The aim of the current study was to determine plasma cortisol and IL-6 concentrations and to investigate whether HPA axis and immune dysregulation is evident at first presentation of children to a sexual abuse clinic. Therefore, based on the retrospective adult data of Kahl *et al.* (2006), we hypothesized that dysregulation of cortisol and IL-6 would be detectable in these children.

Methods

Participants

Adhering to the principles of the Declaration of Helsinki, ethics approval was obtained from the University of Witwatersrand Human Research Ethics Committee (M070724 and M060250). Over six months, eleven female children between the ages of 6 – 12 years, who randomly presented to The Teddy Bear forensic child sexual abuse clinic in Johannesburg, were recruited into the pilot study. The Teddy Bear clinic provides a forensic service to identify whether a child has been abused, both acutely and chronic, and to prepare documentation for court proceedings. However, we have found that the

children often disclose abuse when there are behavioral issues noted by parents or caregivers. Thus, the abuse reported in all the children in our sample was not acute.

The participants were primarily of African descent (African = 10; Indian = 1), comprising female children between the ages of seven and twelve at Tanner stage II or III. The mean age of the sample was 10.8 ± 1.3. Five of the children resided in their own home with a mother, father or grandmother while six children had been placed in care in a children's home because their mother was deceased. All children presented to the clinic with suspected sexual abuse by a stranger. No sexual abuse within the family homes or within the children's home was reported. Forensic results are presented in the demographic table (Table 1). Upon examination by the pediatrician, no infections, fevers or any other illnesses were noted, which were exclusion criteria for this research. Permission for inclusion in the study was granted by either parents or guardians from care facilities.

Venesection and blood handling

To exclude sexually transmitted diseases, investigative blood samples are routinely drawn from the children when there is a high degree of suspicion of sexual abuse. An additional 5ml blood was drawn into a heparinized vacutainer tube (BD-Plymouth) by the same nursing sister to enable cortisol and IL-6 plasma concentrations to be determined. As the forensic examination is an established protocol for the criminal court proceedings there are very specific guidelines which currently do not include psychological assessments for PTSD, depression or anxiety. Additionally, no other

blood tests were done to disrupt the specific operating procedures of the clinic. All blood samples were only taken in the morning between 11.00 and 12.30, immediately following the interview by the pediatrician and a physical examination. Due to the stressful nature of the forensic examination, an increase in plasma cortisol concentration above the expected diurnal rhythm was to be expected (Izawa *et al.*, 2013a; McEwen, 2007). To ensure conformity, after venesection of every patient, only the principle investigator was involved in all the final procedures namely, transfer of the sample to the laboratory for centrifugation (1000xg for 10 minutes at 4°C), aliquoting the plasma, placement of the sample in the freezer (-70°C). Plasma assays for cortisol and IL-6 were batched together and assayed by a qualified medical technician, assisted by the principle investigator.

Cortisol Assay

Cortisol concentrations were determined using competitive immunoassay chemiluminescent technology (Advia Centaur System, Siemens Healthcare Diagnostics). Detection limits were 5.5 – 2069 nmol/l with within-run sample coefficient of variation of <3%.

Cytokine Assay

IL-6 was determined using a Human Cytokine 10-Plex Th1/Th2 assay (Bio-Rad, California, USA; 171-A1001P) and Luminex multi-analyte profiling technology (Bio-Rad, USA) according to manufacturer instructions. Detection limit was <1pg/ml.

Statistical Analysis

All data were analyzed using GraphPad Prism (version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Since there is renewed focus on the increasing need to highlight variance and to stratify both high and low stress responders (Drummond & Vowler, 2011, 2012), our data are presented as scatter plots with mean ± SD. Cytokine concentrations below the detection limit were assigned the lowest detection level. Significance levels were set at $p<0.05$. A student's T-test was used to analyze the difference between groups. A linear regression was conducted to investigate the relationship between cortisol and IL-6.

Results

Demographics

Table 1 shows the demographics of the stratified sexually abused group based on place of residence. All the children presented to the Teddy Bear Forensic Clinic with suspected sexual abuse. At the time of the present study, girls were the predominant patient group at the Teddy Bear Clinic. Moreover, the development of long term psychopathological sequelae following childhood sexual abuse have been noted to be increased in adult female patients (Labudda *et al.*, 2013; Young, & Korszun, 2010).

	Forensic clinic (n=11)	
	Family home (n=5)	Children's home (n=6)
	Suspected stranger abuse, no reported stress in family home	Suspected stranger abuse but no reported abuse in the children's home
Age (mean ±-SD)	11.4±0.62	10.3 ±-1.39
Income	Low	Low
Education	Normal school	Not attending school
Ethnicity		
African	4	6
Indian	1	0
Evidence of sexual abuse		
Conclusive	3	1
Suggestive	2	5
Mother deceased	0	6

Table 1. Demographics of children in the pilot study presenting to the Teddy Bear forensic clinic in a public hospital in Johannesburg, South Africa.

Plasma Cortisol

Figure 1 shows plasma cortisol concentration from all the children. Blood samples were obtained between 11:00 and 12:30, immediately after the medical interview and forensic examination. Preliminary analysis indicated significant variability in the study group (range 102 - 463nmol/l). Further analysis indicated that patients from the children's home were amongst the lower responders, allowing the group to be stratified into those children who resided at home and those children resident in a children's home. There was a significant difference (p=0.0075) in cortisol concentration between the children who resided in a familial home (n=5; 309 ± 101) compared to those who resided in a children's home (n=6; 157 ± 38).

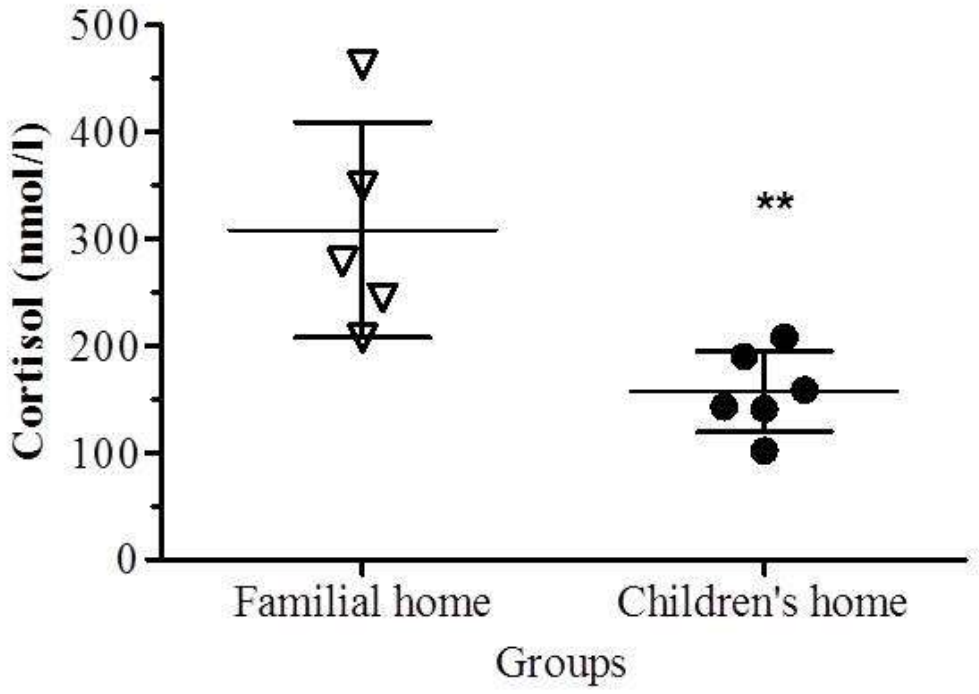


Figure 1. Morning plasma cortisol concentration

Comparison of morning plasma cortisol concentration in children residing in a familial home and children resident in a children's home, after a forensic examination at a sexual abuse clinic in Johannesburg.
** p = 0.0075

Plasma IL-6

Children who resided at home had cortisol concentrations equal to or above 200nmol/l and there was no detectable IL-6 concentration in their plasma sample. In contrast, for the children resident in the children's home (n=6) cortisol concentrations were below 200 nmol/l and IL-6 was detected at a mean concentration of 2.2 ± 2.1 pg/ml for the group (data not shown).

Correlation of Cortisol and IL-6

Figure 2 shows the correlation between plasma IL-6 and cortisol for all the children. A significant negative linear correlation between IL-6 and cortisol existed for the children resident in a children's home (r=-0.8875; p= 0.0183), but not for children that lived with a family member. The residents in the children's home, who had plasma cortisol concentrations below 200nmol/l, show increased IL-6 concentrations. In contrast, in the children residing at home, whose plasma cortisol concentrations were all above 200nmol/l, no correlation is observed.

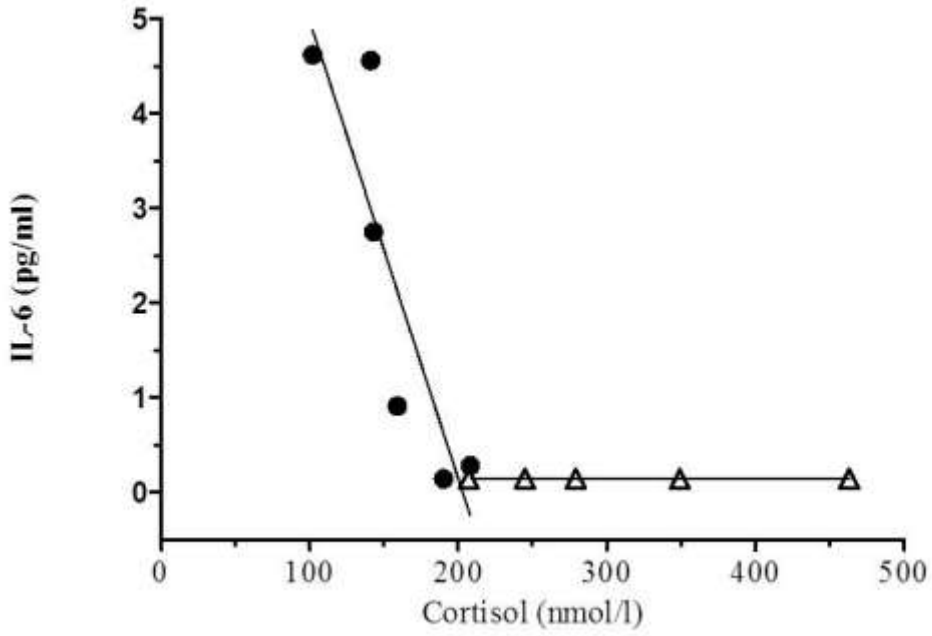


Figure 2. Correlation of IL-6 to Cortisol.

Linear regression of IL-6 to cortisol shows a suppressive effect of cortisol on IL-6 at cortisol concentrations above 200nmol/l.

● Children resident in a children's home

Δ Children resident in their family home

Discussion

The forensic interview and medical examination are considered to be a highly stressful situation for the children. We therefore expected that the children would have an increase in the plasma concentration of the stress hormone cortisol, regardless of the diurnal rhythm, such as occurs in healthy young individuals exposed to a psychosocial stressor (Levine, 2000; Izawa *et al.*, 2013a; McEwen, 2007). Critically, the measurement of cortisol was not a basal morning cortisol concentration but rather cortisol reactivity to a stressor, namely the forensic examination. Differential cortisol responses were noted based on the place of residence. The consequences of this differential cortisol response indicate that the lower the plasma cortisol concentration the higher the IL-6 cytokine concentration in the children from the children's home. In contrast, the relationship between plasma cortisol concentration and IL-6 in the children resident at home showed a suppression of circulating morning IL-6.

The attenuation of the cortisol response to a stressful event parallels the finding by Trickett *et al.*, (2010) of lower morning cortisol concentrations in child victims of sexual abuse. More importantly, Seltzer *et al.*, (2013) have also recently demonstrated that physically maltreated girls have a dampened cortisol response following a social stressor. Kudielka *et al*. (2004) have noted that stress reactivity in response to the TSST is comparable, regardless of the time of day when the test is performed. This further supports our finding that some children failed to mount a stress response since they showed a cortisol level below 200 nmol/l in response to the standardized forensic examination. In particular, our study shows that it is the children resident in a children's

home who present with an apparent lack of an appropriate HPA response when faced with a further stressor, which in this study is the forensic examination. Loman and Gunnar (2010) have noted that children who reside in institutions and children with significant stressors in their family of origin are more likely to present with behavioural and emotional problems associated with a dysregulated stress reactivity pattern. In our study, the children in the institutional setting have demonstrated an attenuated stress reactivity pattern. The serendipitous finding, when the group is stratified by place of residence, of lower plasma cortisol concentration following a stressor suggests an apparent suppression of cortisol secretion in the children resident in the children's home. However, the information that the clinic was given by the caregivers of the home is that the mothers of these children were deceased and that they were found in living as street children in an unsavoury part of Johannesburg.

Thus, the attenuated stress response may not be due to the children's home per se, but rather the additional stressors and traumas such as the loss of primary attachment figures, that these children have experienced (Seltzer *et al.*, 2013). Utilizing a laboratory social stressor with groups of adolescents, Ayer *et al.* (2013) have recently shown that blunted salivary cortisol responses following a stressor are associated with persistent behavioral dysregulation. Moreover, the authors found no sex differences in the attenuated salivary cortisol response (Ayer *et al.*, 2013). The highly significant difference in HPA axis stress response between the two groups of children in our study is in accord with research suggesting that hypocortisolaemia, in response to stress, may

be evident in some patients (Badanes *et al.*, 2011; Heim *et al.*, 2000; Levine, 2000; Trickett, *et al.*, 2010).

Furthermore, as cortisol is a potent anti-inflammatory agent (Raison & Miller, 2003), it is evident that there is a significant impact on the IL-6 cytokine profile when cortisol concentration is above 200nmol/l in the children in our study. The phenomenon of glucocorticoid regulation of the immune system has been highlighted by Raison and Miller (2003). The children resident in a children's home had higher morning IL-6 concentrations during their clinic visit, which were inversely correlated with plasma cortisol concentrations below 200nmol/l. This finding parallels results by Pervanidou *et al.* (2007) who documented an elevated morning IL-6 in children who develop PTSD following a motor vehicle accident. While the increase in IL-6 is modest when compared to IL-6 values in children who are medically ill, the possibility exists that these slight increases, following stressful events, over time have a significant skewing effect on future health outcomes (Raison & Miller, 2011). Increased levels of IL-6 have been associated with psychiatric disorders such as depression (Pace *et al.*, 2006), borderline personality (Kahl *et al.*, 2006) and PTSD (Pervanidou *et al.*, 2007). Our findings indicate that a divergent response to a stressor does occur and abused children respond to the forensic examination stressor as either high or low stress responders (Badanes *et al.*, 2011; Dhabhar, 2009; Heim *et al.*, 2000; Jonkman *et al.*, 2013; Pervanidou, 2008). Previous research that has focused on the behavioural differences between abused and non-abused children has yielded too little information with respect to the long-term pathophysiological implications of divergent cortisol and IL-6

responses in abused children. Thus, we believe that these findings are significant as the cortisol and IL-6 differences between the groups indicates differential priming of their endocrine and immune systems responses, rendering them at risk for different future pathologies which may have either psychiatric or physical outcomes (Dhabhar, 2009).

However, it must be noted that in studying children who present to a child abuse clinic there are significant confounding variables which are frequently not conveyed to the medical staff. Information about verbal or emotional maltreatment, bullying, physical abuse and other traumas such as the death of a family member may not be obtained. The low morning cortisol response to a stressful situation in the children resident at the children's home may be due to the fact that these children have experienced significant other stressors resulting in a complex traumatology and chronic stress (Bowlby, 1952; Dhabhar, 2009; Kaufman *et al.*, 1997; van der Kolk *et al.*, 2005). Importantly, as these were children attending a sexual abuse clinic, the researchers did not want to disrupt the intake process that has been established by The Teddy Bear Clinic. The processes have been developed to reduce any secondary trauma that could be experienced by the child. For this reason the methods to be used for collecting blood samples and swabs from children attending the clinic did not deviate from those already in place. However, given that we have now established that there are significant differences in stress reactivity, it is feasible to consider future evaluation of pre and post examination stress responses.

Furthermore, a limitation of the current pilot study is the lack of an equivalent control group. The stress of the forensic examination is a very specific type of stressor. It is not possible to conduct the forensic interview, which includes a pelvic examination, on non-abused children in this age group. Moreover, a control group within a hospital setting would have resulted in the introduction of other confounding variables, such as responses to physical trauma, medications and infective processes. Therefore, although there is no external control group, the stratification method employed allows for the development of an internal control for each group in this clinical sample. As Kapur *et al.* (2012) have noted, in certain clinical settings, specifically with the consideration of the potential development of psychiatric disorders, it may not be valid to compare a patient group to external controls. While it is known that sexual abuse is associated with long term consequences for some children and not others (Smith *et al.*, 2013), our research provides evidence of differential biological features between sub-groups of children presenting to an abuse clinic (Kapur *et al.*, 2012). Thus, even though there is no external control group we believe that it is important to begin investigation of neuroendocrine and immune differences in this clinical population.

To our knowledge, this is the first study to evaluate both cortisol and cytokine profiles in children presenting to a sexual abuse clinic. Secondly, although our sample size is small, the highly significant difference between the two sub-groups of children indicates that there is a need for increasing our understanding of biological differential features in abused children. Moreover, these results highlight the need for prospective and longitudinal studies into the differential relationship between circulating endocrine and

immune system biomarkers in sub populations of sexually abused children (Kapur *et al.*, 2012). Additionally, to exclude possible spurious attenuation in cortisol release following a stressful event, we would suggest that future studies include the evaluation of adrenocorticotropic hormone (ACTH), the pituitary hormone, responsible for the release of cortisol from the adrenal gland (Carpenter *et al.*, 2007). Thirdly, we present evidence that IL-6 is a significant inflammatory marker when evaluating female children who are experiencing a non-medical stressor. However, longitudinal research is required to determine the impact of differential cortisol and IL-6 concentrations on the children's future ability to maintain hormonal and immune system balance and to prevent neurobiological changes (Dhabhar, 2009; Kapur *et al.*, 2012). Of importance is the possibility that if applicable treatments are started early, during a prodromal stage, there may be a more beneficial outcome for a child or adolescent who has been subjected to severe early life stress (Wachs *et al.*, 2014). Finally, while at the time of this study, young girls were the predominant patient group, there is a need to investigate the biological impact of sexual abuse in young boys.

In conclusion, the current research was undertaken as a "proof of concept" study to investigate cortisol and IL-6 levels in female children presenting to a sexual abuse clinic. Although hampered by a lack of a "normal" control group and a small sample size, our study documented the circulating cortisol and IL-6 levels in children following a forensic examination at a sexual abuse clinic. Moreover, our study indicates that there are physiological differences between groups of such children that may alter their vulnerability to future pathologies. Consequently, due to the known impact of early life

trauma on the later development of long-term psychopathological and somatic sequelae, there is a growing imperative to identify at risk children (Heim *et al.*, 2004). Moreover, in keeping with the suggestion of stratification into more meaningful sub-groups or clusters (Kapur *et al.*, 2013) we recommend that research with abused children consider the need to include biological stress measures to stratify at risk children when they first present following traumatic events. Additionally, there is a growing need to monitor and evaluate these children over the longer-term (Izawa *et al.*, 2013a). Further, the current study established an apparent correlation between circulating cytokine and cortisol levels for different sub-groups of the studied children. Notwithstanding the lack of a non-abused control group, our study highlights the need for comprehensive future studies into physiological changes that may be evidenced at first presentation to a clinic following early life sexual abuse.

CHAPTER 3

Disparate plasma cortisol concentrations in sexually abused female children from Johannesburg, South Africa.

Denise Muller, Sheri-lee Errington, Christopher P Szabo, Neville Pitts and Lorna Jacklin.

Accepted for publication in the Journal of Child Abuse and Neglect

Abstract

Childhood sexual abuse and HPA axis dysregulation have been documented in adult hospitalized patients, diagnosed with Borderline Personality Disorder (BPD). We hypothesized that dysregulation of the HPA axis would be evident at first presentation to a sexual abuse clinic in young girls, between the ages of six to twelve years of age. Blood samples were obtained from female children presenting to a public forensic abuse clinic in Johannesburg and a matched control group of children enrolled in a bone density research project. Compared to the control group (225.5 ± 47.47 nmol/l), plasma cortisol concentrations of the children from the forensic clinic were significantly increased in children who reported abuse by a stranger (324 ± 123 nmol/l), and significantly decreased in children whose histories indicated not only sexual abuse but other family stressors as well (145 ± 14.6 nmol/l). In conclusion, following sexual abuse and a secondary stressor, the forensic examination, there is evidence of diverse responses in two groups of children who present with either a hypercortisolaemia or hypocortisolaemia.

Introduction

Childhood sexual abuse is considered to be a significant risk factor for the development of long term psychiatric disorders such as Borderline Personality Disorder (BPD; Kahl *et al.*, 2006; Rinne *et al.*, 2002, 2003; Zanarini *et al.*, 1997), Post-Traumatic Stress Disorder (Chen *et al.*, 2010; Mathews *et al.*, 2013; Runyon, Deblinger, & Steer, 2014), anxiety (Chen *et al.*, 2010; Mathews *et al.*, 2013) and depression (Chen *et al.*, 2010). Growing research indicates that clinical patients who present with a variety of psychopathologies in adulthood frequently report retrospective associations with a sexual abuse stressor in their formative years (Burghy *et al.*, 2012; Cohen, 2008; Heim et al, 2004). Early life stress has also been associated with priming of the hypothalamic-pituitary-adrenal (HPA) responses (Johnson *et al.*, 2002) and the consequent long-term dysregulation of cortisol responses to stress (Burghy *et al.*, 2012). Plasma concentration of cortisol, the final hormone in the HPA axis cascade, is expected to increase in response to a stressor (McEwen, 2006). However, increased plasma cortisol concentrations are associated with neurodegeneration in the hypothalamus, the hippocampus and neurones in the prefrontal cortex (McEwen, 2006; Wachs *et al.*, 2014).

However, not all children subjected to early life stress present with later life psychopathology (Smith *et al.*, 2013). The possibility exists that a repeat stressor in vulnerable individuals results in different physiological responses than in non-vulnerable individuals (Goodman *et al.*, 2004; Heim *et al.*, 2000). It would also appear that there may be a dichotomous response to stress that would categorize some patients as either high or low stress responders as measured by circulating cortisol in response to a

stressor (Fries *et al.*, 2005; Goldman-Mellor, Hamer & Steptoe, 2012; Heim *et al.*, 2000; Lovallo *et al.*, 2012; Pervanidou, 2008). There is a growing body of evidence that suggests that attenuated cortisol responses to a severe stressor are associated with multiple early life stressors (Goldman-Mellor *et al.*, 2012; Heim *et al.*, 2000; Miller *et al.*, 2013; Raison & Miller., 2003).

Stress which occurs too early in development or during specific developmental stages is considered to prime the endocrine system resulting in long term perturbations (Heim *et al.*, 2004; Johnson *et al.*, 2002; Wachs *et al.*, 2014), rendering a young child at risk for future psychopathologies. Consequently, it would appear critical to determine diagnostic and treatable biomarkers as early as possible following severe early life stress (Wachs *et al.*, 2014). Therefore, as Kapur *et al.*, (2012) note there is a need to identify and stratify at risk children into relevant sub-groups when they first present with a trauma. Currently, however, we cannot identify, using an objective measure such as physiological biomarkers, which children might develop lasting complications from early life stress.

In a small preliminary study on cortisol responses to the stress of a forensic examination at a sexual abuse clinic, in girls (n=11) we found a significant difference in plasma cortisol concentrations in children who lived at home compared to children from a residential care facility. However, our proof of concept study lacked a matched, unstressed control group. Thus, the hypothesis for the current research was that there would be a significant difference in cortisol concentrations in the children from the

forensic clinic when compared to a control group of matched children. The current study has two specific objectives: (1) identify cortisol responses in a matched control group of children under novel basal conditions and (2) identify whether a differential pattern of cortisol response occurs in children whose histories include other stressors in the family home.

Methods

Ethics Approval

Clinical Sample

Adhering to the principles of the Declaration of Helsinki, ethics approval was obtained from the University of the Witwatersrand's Human Research Ethics Committee (M070724 and M060250) for the study to take place at the Teddy Bear Clinic, Charlotte Maxeke Hospital in Johannesburg under the supervision of a pediatrician. The Teddy Bear Clinic, a forensic abuse clinic, identifies the abuse a child has suffered and prepares documentation for court proceedings. As the forensic examination is an established protocol for the criminal court proceedings there are very specific guidelines which currently do not include psychological assessments for PTSD, depression or anxiety. Permission for inclusion in the study was granted by either parents or guardians from care facilities. Prior to the forensic examination the pediatrician informed all children about all the tests that would be conducted including blood tests to evaluate stress responses. Importantly, as noted in Chapter 2, we have found that these children often disclose abuse only when there are behavioral issues noted by parents or caregivers. Thus, the abuse reported in all the children in our sample was not acute.

Control Group

Children enrolled in a bone density study (Meiring *et al.*, 2013) were recruited as a control group. Ethics approval was obtained from the University of the Witwatersrand's Human Research Ethics Committee (M10635). Permission for inclusion in our study was granted by either parents or guardians. The children were informed about the stress study and signed assent forms for inclusion in the research project.

Procedure

Clinical Sample

Seventeen female children, primarily of African descent and between the ages of 6 – 12 years from low income families, presenting to a general hospital forensic child sexual abuse clinic in Johannesburg were randomly recruited into the study. The forensic examination involved a general interview with a qualified intake worker, a forensic medical examination by the pediatrician with specific focus on the genital area to ascertain whether macroscopic evidence of sexual abuse is present, as well as a blood sample (by venesection). In order to exclude sexually transmitted diseases investigative blood samples are routinely drawn from the children at the Teddy Bear Clinic. An additional 5ml blood was drawn into a heparinized vacutainer tube (BD-Plymouth) by the pediatrician to enable cortisol concentrations to be determined. All blood samples were taken in the morning between 10.00 and 12.30, immediately following the interview by the pediatrician and a physical examination. As the forensic examination is an established protocol for the criminal court proceedings there are very specific guidelines thus, no other blood tests were done to disrupt the specific operating

procedures of the clinic." An elevated plasma concentration to a stressor response is expected (McEwen, 2006). In addition, due to the stressful nature of the forensic examination, a further increase in plasma cortisol concentration was to be expected (Izawa *et al.*, 2013).

Control Group

The children from the control group were recruited from a community centre in a low income housing area surrounding Johannesburg (Meiring *et al.*, 2013). The children were matched to the experimental group based on age, ethnicity and socio-economic status. Only children from intact families who were known to have not experienced any recent trauma and were considered to have no behavioral problems were recruited into the control group. Following their Dual energy X-ray absorptiometry scan (DXA; Meiring *et al.*, 2013) children were escorted to an examination room, given refreshments and allowed to watch a film while waiting for the blood sampling procedure. Although some children were visibly stressed by the procedure, their friends were encouraged to stay with them and hold their hand. The blood sample was immediately transferred to the laboratory for centrifugation at 1000xg for 10 minutes at 4°C. Plasma was removed, aliquoted and frozen at -70°C until assayed.

Cortisol Assay

Cortisol concentrations were determined using ^{125}I-labled cortisol solid-phase radiommunoassay (RIA), Coat-A-Count, Human Cortisol (Siemens Medical Solutions Diagnostics, Los Angeles, USA). Detection limits were 0.2υg/dl (5.5nmol/l) with within-run sample coefficient of variation of <5.2%.

Statistical Analysis

Cortisol data were analyzed using GraphPad Prism (version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Data are presented as scatter plots with mean ± SD to demonstrate the significant variance in the sexually abused children (Drummond & Vowler, 2011, 2012). Significance levels were set at $p<0.05$. Data were tested for normality using KS normality test. Based on recommendations to categorize the children based on the type of maltreatment (Kavanaugh *et al.*, 2013), we stratified the data from the children from the Teddy Bear Clinic (see Results, Plasma cortisol). After stratification of the clinical group, an ANOVA with a Newman-Keuls post-hoc test was run.

Results

Demographics

Table 1 shows the demographics of the stratified sexually abused group and the control group. The children in the sexually abused group presented to the Teddy Bear Forensic Clinic with suspected sexual abuse. Upon examination by the pediatrician, no infections, fevers or any other illnesses were noted, which were exclusion criteria for this research. As noted above Kavanaugh *et al.* (2013) suggest stratification based on the type of maltreatment. Thus the children in the sexually abused group were stratified into the following groups: (1) children who were reportedly abused by a stranger but whose parents did not report other stressors in the home (n=11; stranger abuse only) and (2) children abused by a stranger and reports of additional stressors within the family

unit, such as the death of a parent, emotional and/or physical abuse or paternal abuse (n=6; stranger abuse and family stress).

	Clinical Sample (n=17)		Bone density Group (n=14)
	Stranger abuse only (no reported stress in home)	Stranger abuse and family stressors reported in the home	Control group
N	11	6	14
Age (mean ±-SD)	10.4 ± 1.9	9.8 ±-1.9	10.1 ± 0.9
Income	Low	Low	Low
Education	Normal school	Normal school	Normal School
Ethnicity			
African	*10*	5	13
Indian	*1*	1	1

Table 1. Demographics

Demographics of children presenting to the Teddy Bear forensic clinic in a public hospital in Johannesburg, South Africa and children enrolled in a bone density study at the University of the Witwatersrand.

Plasma Cortisol

Figure 1 shows plasma cortisol concentration from the control group of children and those children presenting at the forensic clinic. An ANOVA revealed significant differences between the two groups of sexually abused girls and the control group

(F(2,28)=10.43; p=0.0004). The children from the control group (n=14) responded to the novelty event with a plasma cortisol concentration of 225.5 ± 47.47 nmol/l. Children who were reportedly abused by a stranger but had no other family stressors had a significantly greater mean plasma cortisol concentration (Stranger abuse only; n=11; 324 ± 123 nmol/l). Although the cortisol concentrations in the children who had been abused by a stranger only were normally distributed there was a dominant skewness (1.028). In particular, one child aged 10, overweight and with an apparent history of no stressors in her home life appeared to be the exception in this group with a plasma cortisol concentration of 127.5 nmol/l. (1.6 times the SD from the mean). In contrast, children whose histories indicated stranger abuse and significant family stress (Stranger and family stress, n=6; 145 ± 14.6 nmol/l) presented with attenuated cortisol responses to the forensic examination.

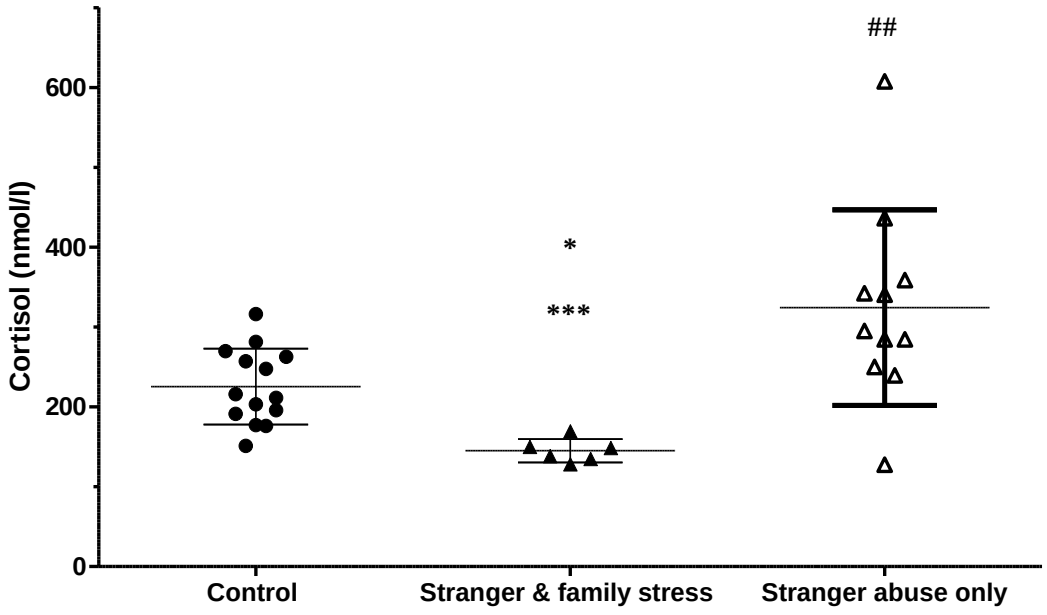


Figure 1. Mean plasma cortisol concentration.

Mean plasma cortisol concentration for the control group following a novel event and for the two groups of sexually abused girls following the forensic examination.

*p<0.05 Control group vs Stranger and family stress

##p<0.005 Control group vs Stranger abuse only

***p<0.001 Stranger abuse only vs Stranger and family stress

Discussion

The visit to the university for the DXA scan for the bone density study was a novelty outing for the children enrolled in the control group. Importantly, under novel conditions, we determined a mean value of 225.50 nmol/l with very little variance for circulating plasma cortisol concentrations in these children. We believe this value represents a valid normal morning value in a group of children from low-socioeconomic backgrounds experiencing a novel event. Venesection may have been a stressor, but the presence of the other children, watching a film and enjoying refreshments after the DXA scan possibly rendered the event less stressful. With respect to our hypothesis of a differential stress response to the forensic examination, it is evident that the sexually abused children may be categorized as either high cortisol responders or low cortisol responders. The stress of the forensic examination is evident in the increased cortisol reactivity in the children who were reportedly abused by a stranger, without any evidence of other home stressors, and may be considered a normal response to this stressful situation. In contrast, the blunted cortisol response by the children who were reportedly abused by a stranger, but have also experienced other severe stressors such as abusive situations in the family or loss of a family member, is evidence of an attenuated stress response. Thus, the dampened cortisol response to the stressful situation in the group of children with additional family stressors is a second significant finding from this study.

Although there can be no direct comparison between the novelty of the DXA scan and the experience of the forensic examination both groups experienced the novelty of attending either the hospital or the university and a potential stressor in the form of the blood draw. Furthermore, to our knowledge it would appear that no direct comparisons of serum cortisol exist in the literature on exposure to a DXA scan. However, Corbett *et al*. (2008) established saliva cortisol responses in normal children who were exposed to a mock MRI scan. Vining and McGinley (1987) determined that a linear correlation of 1-2% exists between serum and salivary cortisol up to serum levels 0f 400nmol/l. Therefore, calculating the possible saliva cortisol concentration, the children experiencing the DXA scan and subsequent blood draw show similar responses to those children experiencing the novelty of a mock MRI scans (Corbett *et al*., 2008).

An increase in cortisol to a stressor is considered a normal response if there is a return to homeostasis (Lovallo *et al.*, 2012; McEwen 2006). Kudielka *et al*. (2004) have noted that stress reactivity in response to the TSST is comparable, regardless of the time of day when the test is performed. Based on this single time point measurement of cortisol we conclude that the increased response to the stressor of the forensic examination is a normal response to a severe stressor. However, the 35% of children in our study whose histories reflect apparently stressful events in their homes have blunted cortisol responses to the stress of the forensic examination. This result is similar to the 24% of children in foster care who reportedly present with atypical blunted salivary cortisol measures (Linares *et al.*, 2008). Furthermore, previous chronic life stress has been shown to result in lower cortisol reactivity (Delahanty *et al.*, 2000; Lovallo *et al.*, 2012;

Miller *et al.*, 2013; Pervanidou, 2008; Yehuda, McFarlane, & Shalev, 1998). Moreover, chronic childhood maltreatment has been associated with complex post-traumatic stress disorder which has been proposed to account for the complicated symptomatology evident in some children (Leenarts *et al.*, 2013). In sexually abused adolescent girls, Keeshin *et al.* (2013) have shown that young girls who present with PTSD have blunted cortisol awakening responses. Interestingly in their study they demonstrate that previous childhood adversity is associated with an increased severity of PTSD (Keeshin *et al.*, 2013).

Low socio-economic status is considered to be a chronic stressor and has also been associated with divergent morning salivary plasma cortisol levels (Cutili *et al.*, 2010). In their study, samples were obtained from children aged between four and seven years of age following three cognitive tasks. The greater the number of negative lifetime events experienced by each child was shown to result in an attenuated cortisol response to the tasks (Cutili *et al.*, 2010). Similarly, all the children in our study were from a low socio-economic environment, but the children that presented with the attenuated plasma cortisol response were the children who had experienced other significant stressors such as abuse or maternal loss in their family home. Thus, our research supports the evidence that sexually abused children with histories of other family stressors show an attenuated cortisol response to stress (Leenarts *et al.*, 2013). Good maternal care may be a significant ameliorating factor in preventing attenuated cortisol responses during stressful events (Tang *et al.*, 2013).

Of concern is that, in the group "stranger abuse only", one child presented with an apparently atypical blunted cortisol response which we believe needs further consideration. The 10 year old patient was referred to the forensic clinic from a general hospital where she was treated for Human Papilloma Virus (HPV) warts in her mouth. A review of her history revealed that she was the only overweight child in the study and that she denied any sexual abuse. One of the reasons for the lack of a definitive history of family abuse or stress may be that the personnel were unable to ascertain with any certainty that the parents knew of or disclosed all the abuse that the child may have suffered. Although our stratification method was to stratify based only on type of abuse (Kavanaugh *et al.,* 2013), in the future it may be important to follow Kapur et al's (2012) recommendation to stratify the sample based on a biological value such as the attenuated cortisol response. Yehuda *et al.* (1998) also suggest that this out of the ordinary response requires further attention.

In the current study, our data suggest that the stratification should be based on patients below one standard deviation of the mean of the control group. An attenuated response to a stressor may have further health implications. Insufficient glucocorticoid signaling has been associated with impaired control of the immune system resulting in increased inflammatory responses (Raison & Miller, 2003). Pervanidou *et al.* (2007) note that children who present with PTSD following a motor vehicle accident have increased interleukin-6 (IL-6) responses in the morning and elevated salivary cortisol responses in the afternoon. It is also possible that, over the longer term, the children who present with attenuated cortisol responses to the forensic examination stressor in the morning

may have an altered diurnal cortisol trajectory possibly with a delayed cortisol response (Karlamangla *et al.*, 2013). Kahl *et al.* (2006) have noted that, following the retrospective reporting of early life sexual abuse, dysregulation in cortisol and the immune system are evident in adult patients diagnosed with BPD.

As the development of Borderline Personality Disorder has been associated with childhood stressors and the experience of abuse and neglect are commonplace in 91% of the patients diagnosed with BPD in later life (Gratz *et al.*, 2008; Zanarini *et al.*, 1997) it is clear that longitudinal studies are needed to elucidate the long term physiological impact of abuse, and sexual abuse in particular. Moreover, we would recommend that future studies include the evaluation of adrenocorticotropic hormone (ACTH), the pituitary hormone, responsible for the release of cortisol from the adrenal gland (Carpenter *et al.*, 2007). Thus, a limitation of the current study is the cross-sectional prospective nature of the research. As these were children attending a sexual abuse clinic, the researchers did not want to disrupt the intake process that has been established by The Teddy Bear Clinic. The processes have been developed to reduce any secondary trauma that could be experienced by the child. For this reason the methods to be used for collecting blood samples and swabs from children attending the clinic did not deviate from those already in place. However, as noted previously, given that we have now established that there are significant differences in stress reactivity, it is feasible to consider future evaluation of pre and post examination stress responses. Moreover, it is possible that, due to the diurnal nature of cortisol secretion with a higher peak in the morning, the measurement of cortisol reactivity in the morning may result in lower

cortisol reactivity in the morning (O'Leary *et al*., 2007). However, Kudielka *et al*. (2004) have noted that stress reactivity in response to the TSST is comparable, regardless of the time of day when the test is performed. This further supports our finding that some children failed to mount a stress response since they showed a cortisol level below 200 nmol/l in response to the standardized forensic examination.

To fully investigate the clinical importance of these divergent findings and the long-term impact of both the increased and the attenuated cortisol concentrations in the sexually abused girls, there is a need for a more comprehensive longitudinal study (Chen *et al.*, 2010; Shenk *et al.*, 2010). In particular, we would recommend evaluation of diurnal salivary cortisol measures which include a cortisol awakening, morning, afternoon and evening measures, in the days following the forensic examination. Furthermore, once the court proceedings are completed, we would recommend personality and PTSD questionnaires to determine whether there are any correlations between possible long term PTSD development and personality disorders and the observed differential cortisol responses (Goodman *et al.*, 2004). However, it must be noted that many of our children are only fully conversant in their own home language, which may be any one of 11 official languages, resulting in a dearth of psychometric tests that have been validated in these languages for use with our clinical sample.

In conclusion, this study is timeous and beneficial for a number of reasons. Firstly, it is evident from our ongoing research with sexually abused young girls that significant physiological differences are detectable at an early age. Moreover, these physiological

differences are correlated with their current level of abuse, neglect or distress. This study confirms findings from our preliminary research, and from research in adult patients (Goldman-Mellor *et al.*, 2012; Heim *et al.*, 2000) of divergent responses to a stressful situation. Research into physiological dysregulation at the earliest time point in a child's life may be important to ameliorate the long term psychological effects such as personality disorders and affective instability (Etter *et al.*, 2013; Heim *et al.*, 2008). Secondly, we have determined a possible "normal" basal plasma concentration of 225.50 nmol/l with a small variance for children from a low socioeconomic group experiencing a novel situation. Thirdly, we have highlighted the need for stratification of clinical groups based on the homogenous nature of their biological response to a stressor. In this clinical population of children, the dichotomy between high and low cortisol responders is evident. The finding of medical biomarker such as cortisol response to a stressor to identify children at greater risk for trauma sequelae is critical (Binder & Holsboer, 2012; Walsh *et al.*, 2013). Thus, based on our data, cortisol reactivity should be the differentiating feature and we would recommend that clinical stratification should be based on whether the patient presents with either hypercortisolism or hypocortisolism. These findings have implications for the establishment of effective diagnostic and treatment programs and consequently for recovery and prevention of long-term medical sequelae (Daskalakis *et al.*, 2013a). Moreover, we believe these results will enable the implementation of a prospective study of circulating endocrine biomarkers in sexually abused children.

Acknowledgments

We would like to thank Sister Portia Clark and all of the staff at the Teddy Bear Clinic, Charlotte Maxeke Hospital in Johannesburg. This work was supported by the Iris Ellen Hodges Trust – Stress and Emotional Problems Grant to Dr Lorna Jacklin.

CHAPTER 4

VARIATION OF MATERNAL DEPRIVATION PROTOCOL REVEALS INCREASED BODY MASS AND VISCERAL FAT MASS IN FEMALE SPRAGUE DAWLEY RAT PUPS**.**

Denise Muller**,** Christopher P Szabo, Neville Pitts

.

Submitted to Journal of Stress

Abstract

Associations between early life stress and later life dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and the immune system have been found in adult patients and in animal studies. In a pre-clinical rodent model of early life stress, the current study assessed whether a relationship between maternal deprivation and biomarkers of stress and inflammation are identifiable at an early developmental time point namely, weaning on post-natal day 21 and following the stress of forced weaning, twenty-four hours later. We assessed the impact of the maternal deprivation stress on circulating corticosterone, interleukin-6, white blood cell count and body mass. Importantly, increased body mass and fat mass were significantly correlated with corticosterone and the immune system markers after forced weaning. Specifically, our data reveal that maternal deprivation into the third week of neonatal life results in an attenuation of corticosterone following a second early life stressor namely, weaning. Moreover, this protocol is associated with an increase in visceral fat mass and body mass that merits further investigation. Thus, this research provides evidence for the occurrence of early physiological dysregulation following maternal deprivation.

Introduction

Early life stress and mother – infant relationship difficulties in human infants are linked to a variety of later life pathologies, such as attachment disorders (Bowlby, 1987a, 1987b; Raineki *et al.*, 2010), anxiety and depression (Hayley *et al.*, 2005; Heim *et al.*, 2008, 2010), personality disorders (Kahl *et al.*, 2006) and post-traumatic stress disorder (PTSD; Jovanovic, et.al., 2009). Moreover, HPA dysregulation has been found in patients with depression (Penninx *et al.*, 2013), personality disorders (Kahl *et al.*, 2006) and PTSD (Pervanidou & Chrousos, 2012b). Similarly, in validated pre-clinical rodent models of maternal deprivation, HPA axis activation has been found to be dysregulated following early life stress (Levine, 2005). In humans and animals, following early life stress, responses to further stressors have been found to result in either an increase in glucocorticoid secretion (Dhabhar & McEwen, 2007; Levine 2005; Pervanidou & Chrousos, 2012b) or a decline in glucocorticoid secretion, dependent on the timing and nature of the later stressor (Fries *et al.*, 2005; Gustafsson *et al.*, 2010; Heim *et al.*, 2000; Heim *et al.*, 2010; Levine, 2005; Pervanidou, 2008). This bi-directional nature of the HPA axis response to a stressor may further result in either immune enhancing or immunosuppressive effects (Beitia *et al.*, 2005; Dhabhar, 2009).

Immune system dysregulation, as measured by increases in immune system messenger molecules, has also been noted in patients who present with depression (Anisman *et al.*, 2002; Anisman *et al.*, 2005), in children and adolescents with Post Traumatic Stress Disorder (PTSD) (Pervanidou, 2008), in adult clinical patients with co-morbid depression (Anisman, 2009; Raison *et al.*, 2006; Raison & Miller, 2011) and in patients

with disrupted early relationships who present with Borderline Personality Disorder (BPD; Kahl *et al.*, 2006). Similarly, in animal models the relationship between stress and immune system dysregulation has been documented (Anisman *et al.*, 2005; Connor *et al.*, 2005). Due to the ubiquitous nature of Interleukin-6 (IL-6), a pro-inflammatory messenger in the immune system, it is considered to be a significant biomarker in human psychopathology (Kahl *et al.*, 2006; Pervanidou, 2008). Following maternal deprivation and a later life stressor, increased levels of IL-6 have also been documented in colonic secretions in adult rats (O'Mahony *et al.*, 2009; O'Malley *et al.*, 2011). Furthermore, nociceptive pain responses in female rats following maternal deprivation are associated with increased levels of IL-6 in the hippocampus (Burke *et al.*, 2013). Although changes to the pro-inflammatory cytokine milieu, specifically IL-6, are evident in later stages of development, the possibility exists that other innate immune system molecules such as macrophages may provide more promising data at an early developmental age (Dantzer & Kelley, 2007; Dhabhar *et al.*, 2012; Khanfer *et al.*, 2010; Miller, Maletic & Raison, 2009).

Furthermore, documented links exist in adult psychiatric patients with somatic consequences, such as obesity and metabolic syndrome, who retrospectively report early life stress (Boynton-Jarret *et al.*, 2012; Jahng, 2011; McIntyre *et al.*, 2007; Pennix *et al.*, 2013; Shelton & Miller, 2010, 2011). HPA axis perturbations (Kyrou & Tsigos, 2009; Ryu *et al.*, 2008; Spencer & Tilbrook, 2011) and immune system dysregulation (Black, 2003; Bugge, *et al.*, 2012; Shelton & Miller, 2011) have been noted in obese humans and animals. Moreover, the use of childcare facilities to allow mothers to return to work

for economic reasons could have an impact on children which may only become evident later in life (Belsky, 2001). However, the aetiological relationship between HPA axis, immune system, and varying degrees of dysregulation and imbalance during early development has been difficult to establish in human children and has not been fully delineated in pre-clinical models of early life stress (Faturi *et al.*, 2010; Litvin *et al.*, 2010; McEwen, 2000; McEwen, 2003).

In summary, extensive research into early life stress has demonstrated an association between early life stressors and long term affective and somatic disruption noted in patients and animal models (Heim *et al.*, 2010). Notwithstanding the evidentiary data of neurobiological and behavioral impacts following early life stress, there remains a gap in our knowledge regarding possible circulating markers at early developmental time points that may account for the etiology of later pathologies (Litvin *et al.*, 2010). Thus, we have hypothesized that maternal deprivation, as an early life stressor, would be detectable in rats, at weaning. Firstly, we examined the relationship between circulating corticosterone and IL-6, white cell counts, fat mass and body mass on the day of weaning (post natal day 21). Secondly, to investigate the stress of forced weaning, in another cohort of pups, we examined the association of circulating corticosterone and IL-6 and body mass twenty-four hours after weaning (post natal day 22). This study therefore documents the first phase of a long-term developmental study. We report here the impact of maternal deprivation on HPA activation, plasma IL-6 concentrations, white cell counts and body and fat mass in the female pups on PND 21 and PND 22.

Methods

Animals

Twenty-four Sprague-Dawley female rats and their offspring were bred in the University of Witwatersrand Central Animal Service. To achieve a pregnancy, females obtained from our in-house breeding program were placed with a male Sprague-Dawley rat for five days. The females were checked daily for a vaginal plug. In order to minimize any further ante-natal stress, as soon as a successful mating had occurred the dams were group-housed (2-4 animals per cage) and remained undisturbed in a designated pre-natal area of the animal unit. The principal researcher was responsible for all care of the animals from this point. Three days prior to the expected delivery date the pregnant females were separated and placed in individual 47 x 25 x 21 cm3 clear plastic cages. The nursery was maintained on a 12/12 h light/dark cycle with lights on at 06h00. Controlled ambient temperatures were 24°C, with humidity at 55%. Standard rat chow and water were available ad libitum. The day of delivery was designated post-natal day (PND) 0. Post-delivery, each litter was housed with its own dam until forced weaning on PND 21. Litters were not culled or cross-matched to different dams to avoid any confounding stressors. Dams and pups were left undisturbed until PND seven (PND 7) after which a regular bi-weekly litter changing strategy was implemented. Litters were weaned, sexed, separated and weighed on PND 21. All experimental procedures were approved by the University of the Witwatersrand Animal Ethics Committee (certificate 2005/74/6), consistent with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals revised 1996.

Experimental Design

Maternal deprivation. Maternal deprivation (MD) is a well validated stress procedure (O'Mahony *et al.*, 2009; Veenema, 2009). In order to investigate the impact of the stress that young infants incur during early life child care (Belsky, 2001), our maternal deprivation protocol was a variation of the standard protocol and was implemented from PND 7 – 20. Dams were randomly assigned to either the maternal deprivation (MD) group or the control group (NMD). Dams from the MD group were separated from their pups for three hours daily between 09:00 – 12:00 each day. The dams were removed from the pups and placed in a soundproof room ten metres away from their pups. In contrast, the non-maternally deprived (NMD) pups remained undisturbed with their dam.

Blood collection. On PND 21, the day of weaning, between 09:00 – 11:00, a cohort of female pups were weighed and killed from each of the control (NMD, n=12) and experimental groups (MD, n=11) respectively. The nadir of murine corticosterone secretion occurs during the lights on period in nocturnal animal species (de Boer & van der Gugten, 1987). Blood (2-3 mls) was obtained by cardiac puncture within four minutes of anesthesia, as per the recommendations of Arnold and Langhans, (2010). Following cardiac puncture animals were not revived but were killed using 200mg per kg intra-cardiac sodium pentobarbitone (Kyron Laboratories, Benrose, RSA). The remaining pups were weighed and group housed in either a two or three same-sex cage, maintained on the same light/dark cycle and at the same temperature and humidity as the nursery. On PND 22 (24 hours post weaning) a second cohort (n=10) of animals from

both groups was killed by the same procedure described above. Blood was collected in a heparinized tube and centrifuged at 1000xg for 10 minutes at 4°C. Plasma was aliquoted and stored at -80°C for later determination of plasma corticosterone and cytokine concentrations.

Analyses

Corticosterone determination. Plasma corticosterone concentrations were determined, for both PND 21 and PND 22, on all blood samples. A rat double-antibody 125I-corticosterone radioimmunoassay kit (MP Biomedicals, Orangeburg, NY) was used as per manufacturer's instructions. The lower detection limit for the corticosterone kit was 7.7 ng/ml. Inter-assay variability for the assay was less than 6.9% while intra-assay variation was less than 7.3%.

IL-6 cytokine analysis. Plasma IL-6 cytokine determinations for the NMD and MD pups, for both PND 21 and PND 22, was conducted using the BioSource Rat IL-6 Elisa (BMS625, Bender Medsystems, Austria). The lower detection limit for the kit was 8 pg/ml. Inter-assay variability for the assay was less than 10% while intra-assay variability was less than 5%.

WBC counts. As the experiments were done in specific phases, white blood cell counts for PND 21 were evaluated manually by diluting 40µl of whole blood with 360µl Turks solution (1ml Gentian violet 1% aqueous solution and 2ml glacial acetic acid in 100 ml distilled water) to haemolyse red blood cells and increase visualization of the leukocytes.

The diluted blood solution was added to a Neubauer Improved hemocytometer chamber (Electron Microscopy Sciences, Set Point Instruments, South Africa) and incubated for three minutes at room temperature prior to counting the WBC's using a 10x objective on the microscope.

Body mass and fat mass determination. Body weight (g) was measured for all animals prior to aneasthetization. In order to evaluate whether differences in body mass was related to fat stores and not muscle mass, visceral fat, including perirenal deposits, from each rat pup was dissected out, blotted dry and weighed (Gerbaix *et al.*, 2010). Fat mass was expressed as a percentage of body weight.

Statistical Analysis

All data were analyzed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Corticosterone and body mass from the NMD and MD groups on both PND 21 and PND 22 were analyzed using a one way analysis of variance (ANOVA) and normality was confirmed with the D'Agostino and Pearson omnibus test. Normally distributed data was subjected to the Newman-Keuls Multiple Comparison post-hoc test at $p<0.05$. As the IL-6 cytokine concentrations were not normally distributed, even after a natural log transformation, data are presented as median and range and were analyzed using a Kruskal-Wallis with Dunn's post-hoc test. White blood cells, body mass and fat mass between the groups NMD and MD on PND 21 were assessed for normalcy with D'Agostino and Pearson omnibus test. This data was then compared with ANOVA or Student's t-test where

applicable. Further analyses using linear regression were conducted to investigate relationships between specific variables. Statistical significance was accepted at $p<0.05$. In accordance with concerns raised regarding the inapplicability of focusing primarily on means in clinical populations and the increasing need to highlight variance and stratify both high and low stress responders, where necessary, our data are presented as scatter plots (Drummond & Vowler, 2011, 2012).

Results

Maternal deprivation: physiological impact on the pups

Plasma corticosterone. Figure 1 shows the plasma corticosterone concentration from blood collected by cardiac puncture on PND 21 and from a second cohort of animals on PND 22. Note that the animals killed on PND22 had experienced 24 hours without their dams (post weaning). ANOVA indicated that the means between the groups were significantly different ($F(3,39) = 3.91$, $p=0.0156$). There was a significant increase in plasma corticosterone concentration (ng.ml-1) in the NMD group from PND-21 to PND-22 ($q(16) = 3.89$, $p<0.05$). On PND-22, the NMD group had a significantly higher plasma corticosterone concentration compared to the MD group ($q(15) = 3.50$, $p<0.05$).

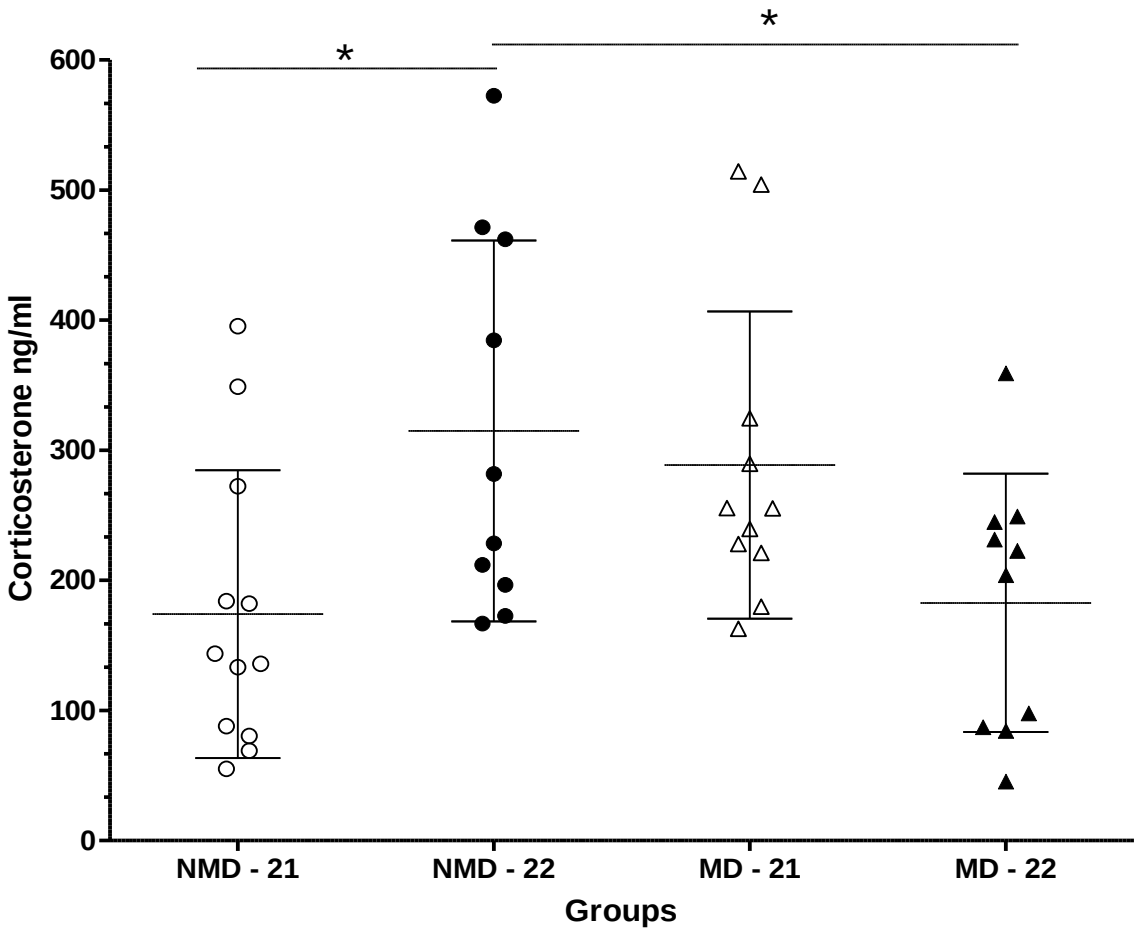


Figure 1. Plasma corticosterone concentrations

Plasma corticosterone concentrations (mean ± SD) presented as a scatterplots for the groups NMD and MD on the day of weaning (PND21) and 24 hours post weaning (PND 22). NMD = non-maternally deprived, MD = maternally deprived.

* $p<0.05$

Interleukin-6. Figure 2 shows the plasma IL-6 concentration on PND 21 and on PND 22 from a second cohort of pups. No significant differences were found between the groups of pups on either day.

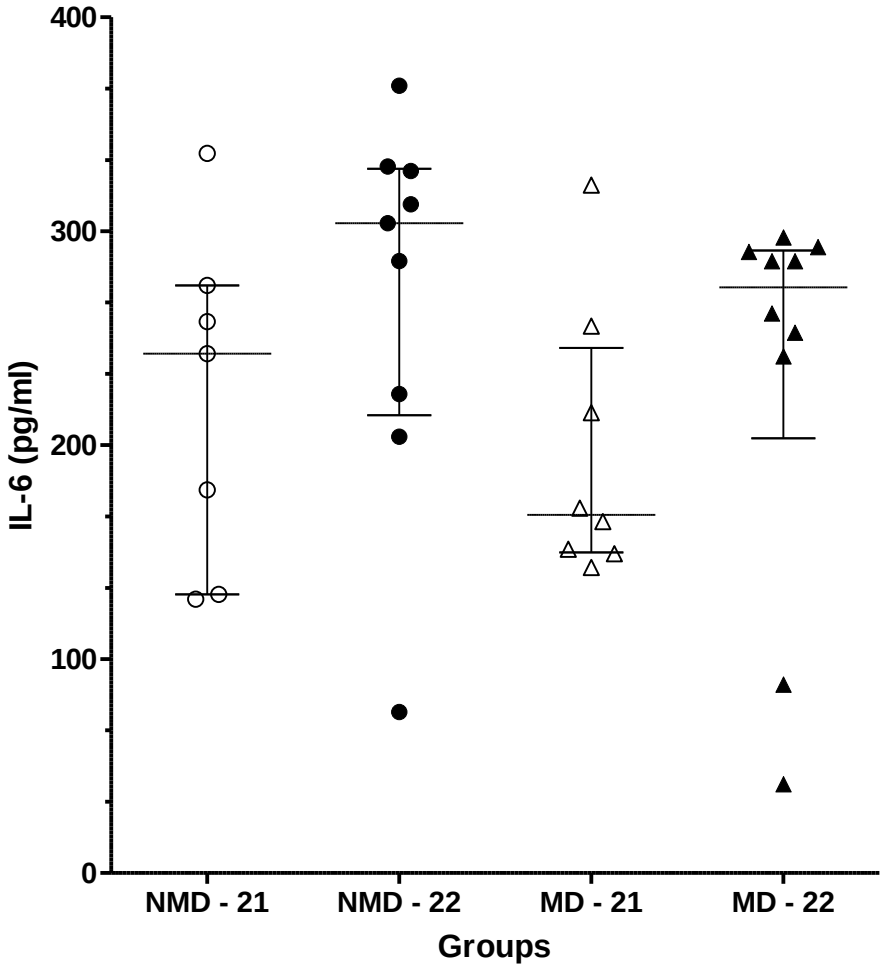


Figure 2. Plasma IL-6 concentrations

Plasma IL-6 concentrations presented as a scatterplots (median and interquartile range) for the groups NMD and MD on the day of weaning (PND21) and 24 hours post weaning (PND 22).

NMD = non-maternally deprived, MD = maternally deprived.

White blood cell counts. Figure 3 shows white blood cell (WBC) counts on PND 21. On PND 21 there is a significant increase in WBC in the maternally deprived group ($t(21)=2.25$, $p=0.0351$). Of note, the variance of white blood cells is significantly different between the groups ($F(10,11)=13$, $p = 0.0002$).

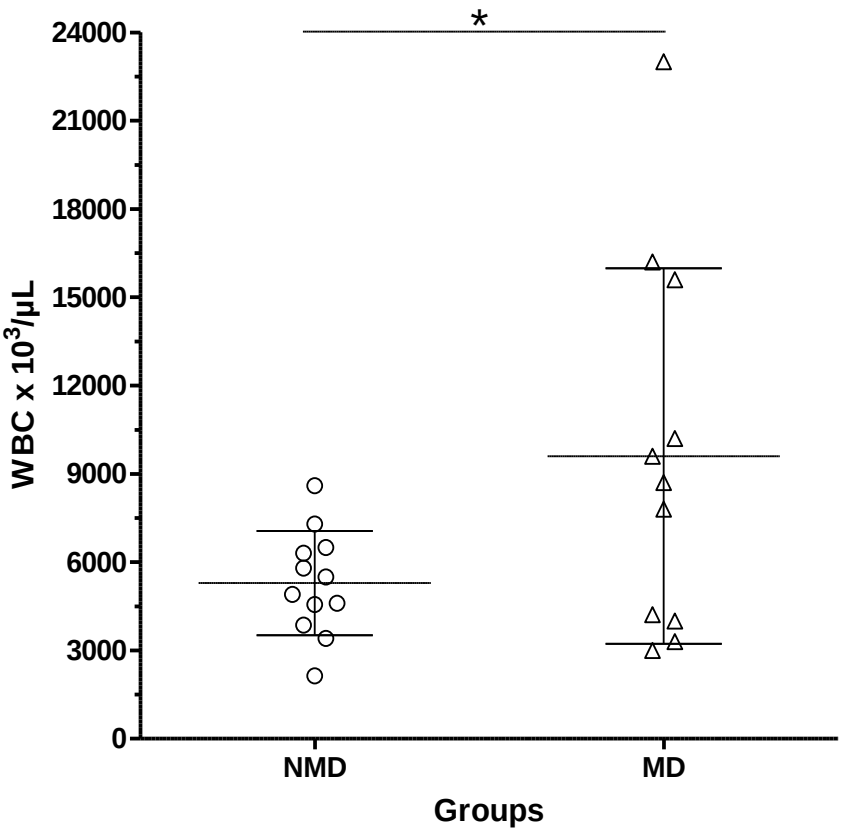


Figure 3. White blood cell counts
White blood cell counts (mean ± SD) on PND 21 in NMD (n=12) and MD (n=11) rat pups.

Body mass. Figure 4 shows the comparison of body mass (mean ± SD) between the NMD and MD groups on PND 21 and on PND 22. ANOVA indicated that the means between the groups were significantly different ($F(3,39) = 5.70$, $p = 0.0025$). On PND 21, the means are significantly different with a significant greater body mass in the MD group when compared to the NMD group ($q(17) = 4.612$, $p < 0.05$). On PND 22 the NMD group had a significantly lower body mass than the MD group ($q(15) = 3.478$, $p < 0.05$).

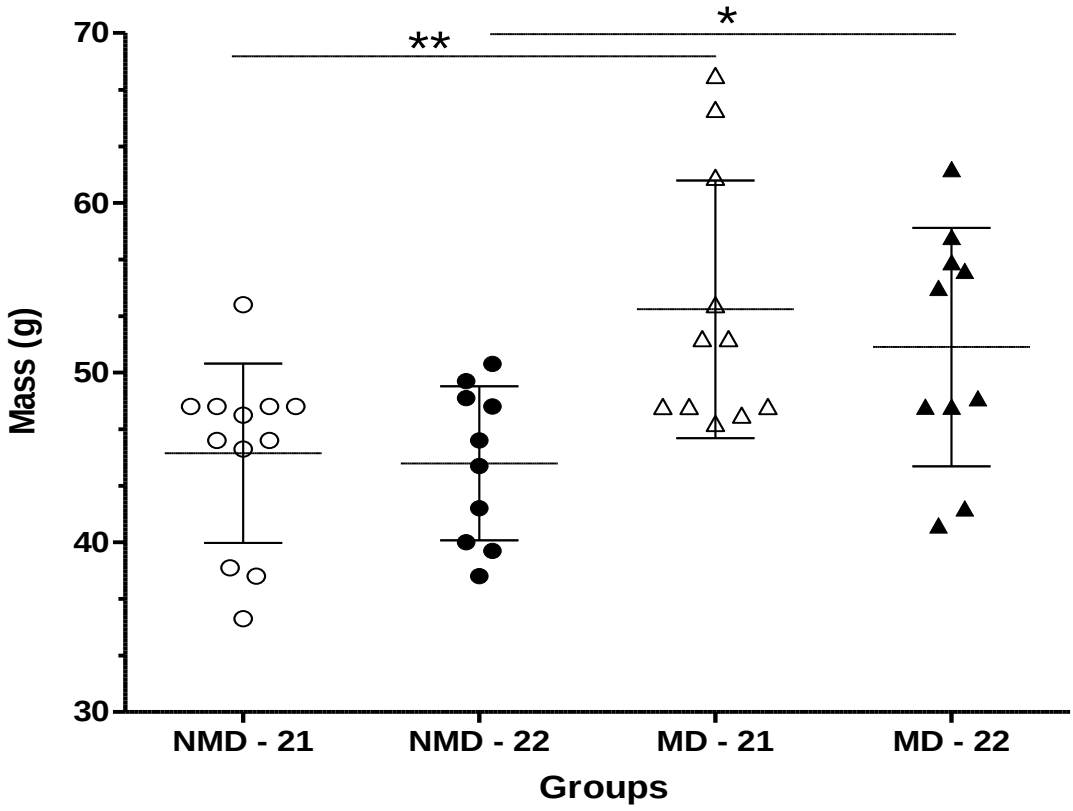


Figure 4. Body mass

Body mass presented as a scatterplot for the day of weaning (PND 21) and twenty-four hours post weaning (PND 22) for the NMD and MD groups.

NMD = non-maternally deprived, MD = maternally deprived.

** $p < 0.01$, * $p < 0.05$

Fat mass. Figure 5 shows the comparison of fat mass expressed as a percentage of body mass, between the NMD and MD groups on PND 21. Fat mass expressed as a percentage of body mass was found to be significantly lower in the NMD when compared to the MD group ($t(21)=2.55$, $p=0.0186$).

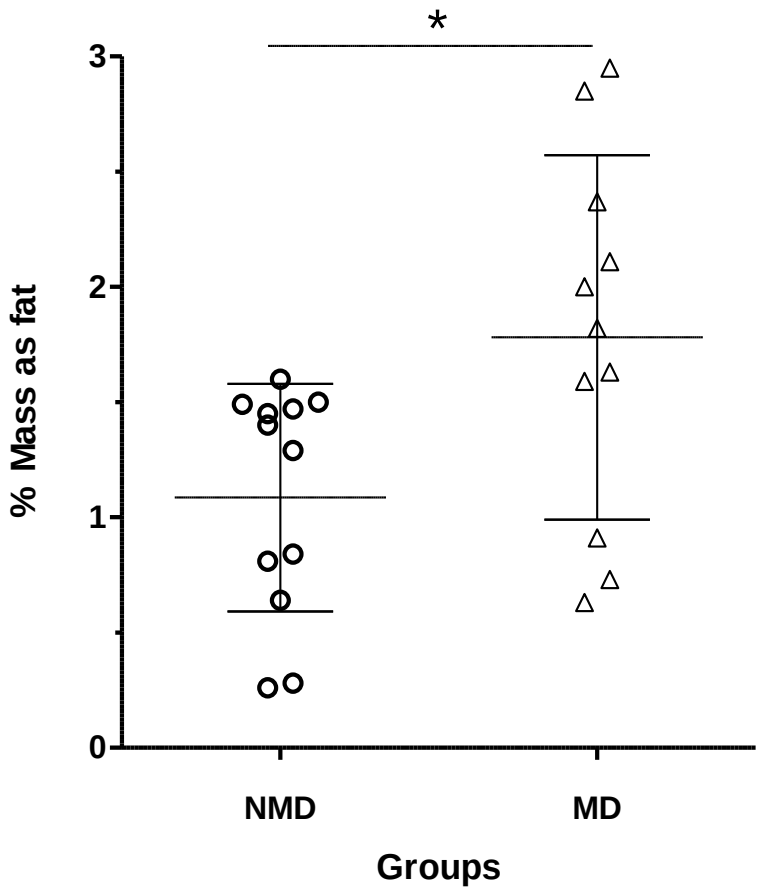


Figure 5. Visceral fat expressed as a percentage of body mass.
Visceral fat expressed as a percentage of body mass for rat pups on the day of weaning (PND21). MD rat pups had significantly more fat as a percentage of body mass than the non-maternally deprived pups.
NMD = non-maternally deprived, MD = maternally deprived.
*p=<0.05.

Further investigations

Given the increase in body mass and the increase in fat mass as a percentage of body mass in the MD group, we wanted to establish if there was any hormonal or immunological correlation to the change in body mass. Therefore we applied linear regression analysis to body mass and the biological markers corticosterone, IL-6 and WBC counts.

Corticosterone / body mass correlation. Figure 6 shows corticosterone by body mass correlation for the NMD and the MD deprived neonate pups on PND 22. On PND 21 there is no correlation between body mass and corticosterone for the NMD pups ($r(1,10)=0.24$, $p=0.46$) (data not shown). Post-weaning on PND 22 there is a significant negative correlation between body mass and corticosterone for the NMD pups ($r(1,8)=-0.71$, $p=0.02$). In the MD pups the relationship between plasma corticosterone concentration and body mass shows a positive relationship between corticosterone and body mass ($r(1,8)= 0.81$, $p=0.005$), indicating that the heavier the pup, the higher the plasma corticosterone concentration.

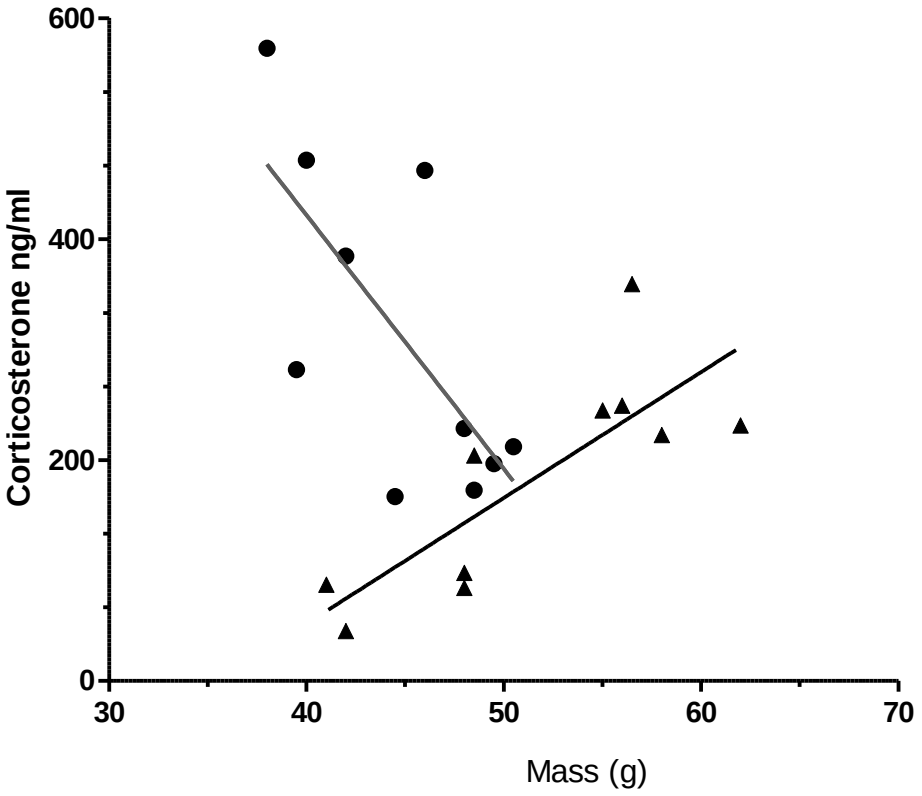


Figure 6. Linear regression between corticosterone and body mass

Linear regression between corticosterone and body mass twenty-four hours post weaning (PND 22) illustrates the significantly different relationship between the corticosterone and body mass. In the non-maternally deprived (NMD, ●, n=10) pups there is a negative correlation between body mass and corticosterone following weaning. The maternally deprived (▲; n=10) pups show a positive correlation between corticosterone concentration and body mass following 24 hours without the dam.

NMD = non-maternally deprived, MD = maternally deprived.

IL-6 / body mass correlation. Figure 7 shows corticosterone by body weight for the NMD and the MD deprived neonate pups on PND 22. No significant relationship was evident for IL-6 and body mass on PND 21 (data not shown). On PND 22 no relationship exists between body mass and IL-6 for the NMD pups ($r(1,7)=0,39$, $p>0.5$). However, there is a significant positive relationship between body mass and IL-6 for the MD pups($r(1,8)=0.76$, $p=0.01$).

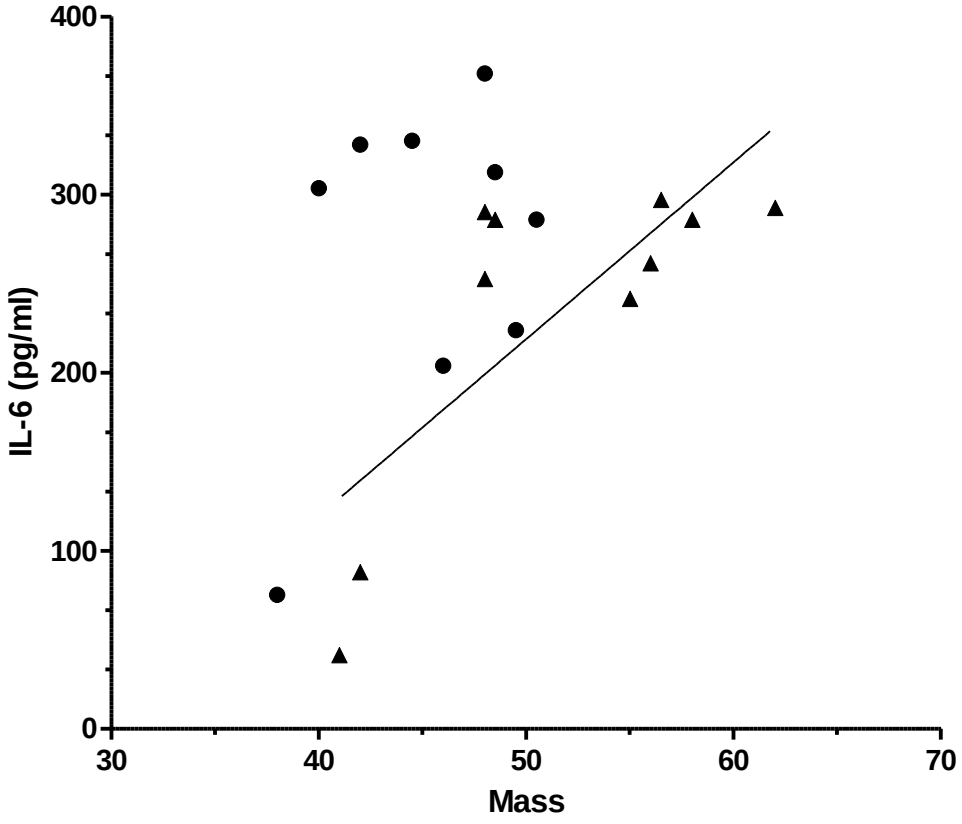


Figure 7. Linear regression between IL-6 and body mass
Linear regression between IL-6 and body mass twenty-four hours post weaning (PND 22) illustrates the relationship between IL-6 and body mass in NMD (●, n=10) pups and MD(▲; n=10) pups.
NMD = non-maternally deprived, MD = maternally deprived.

WBC count / body mass correlation. Figure 8 shows WBC by body mass for the NMD and the MD neonate pups on PND 21.There is no significant relationship between white blood cell count and body mass in the NMD group ($r(1,10) = 0.53$, $p=0.08$). The MD group shows a significant positive relationship between white blood cell count and body mass on PND 21 ($r(1,9) = 0.87$, $p=0.0004$).

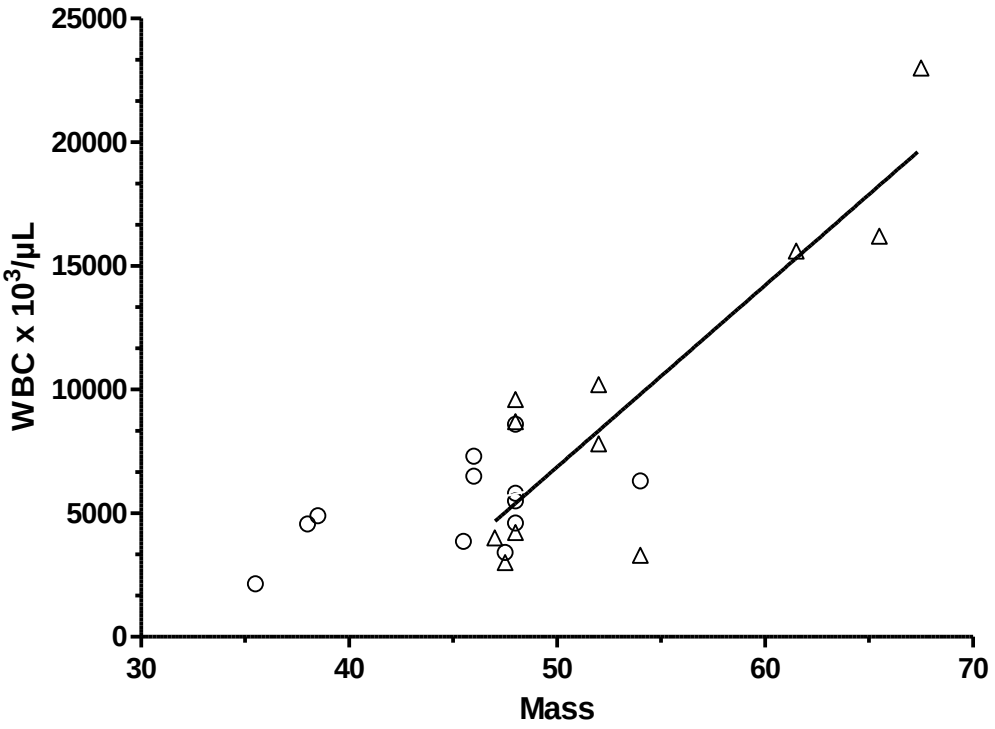


Figure 8. Linear regression, on the day of weaning, between white cell count and body mass.

Linear regression, on the day of weaning (PND 21), between white cell count and body mass illustrates the non-significant relationship between body mass and white blood cell count in the NMD group (○, n=12). In the MD group a significant positive relationship between body mass and white blood cell count is evident (Δ, n=11).

NMD = non-maternally deprived, MD = maternally deprived.

Discussion

The present study provides evidence for early detection, in a rat model, of the physiological impact of maternal deprivation stress into the third week of neonatal life. By PND 21, our maternal deprivation protocol causes an increase in body mass and visceral fat mass in the rat pups. Furthermore, on PND 22, following the stress of weaning, MD pups show reduced activation of the HPA axis, as measured by circulating corticosterone. In these maternally deprived rat pups we also show significant positive correlations between the HPA axis stress response and body mass. Immunologically there is also a positive correlation between WBC and body mass and between IL-6 and body mass in these pups. Importantly, our study demonstrates that identifiable corticosterone and immune system dysregulation are correlated with increases in body mass early in development.

A significant increase in plasma corticosterone, indicative of a stress response to the removal of the dam, is evident in the NMD pups twenty four hours post weaning. The expected increase in plasma corticosterone post weaning confirms that forced weaning is a stressor to pups that have not experienced maternal deprivation. In contrast, on PND 22, the MD pups have a significantly lower plasma corticosterone than their NMD controls. The lack of an increase in corticosterone 24 hours post weaning in the MD deprived pups may be indicative of adaptation to the frequent removal of the dam. Of note, the maternally deprived pups were also significantly heavier than the pups in the non-maternally deprived group. In the maternally deprived pups, the significant increase in body mass is detectable as early as PND 21.

Furthermore, on PND 22 there are differentially significant correlations between plasma corticosterone and body mass in both the maternally deprived and non-maternally deprived pups. In the non-maternally deprived group the negative correlation with body mass indicates that the lighter pups present with increased levels of corticosterone concentrations following 24 hours without the dam, whereas the heavier pups in this group have a lower plasma corticosterone concentration. The observed negative correlation between body mass and corticosterone in the NMD pups may indicate that the lighter the pups are, the more severe the nutritional deprivation and associated stress, resulting in the need to maintain a higher plasma corticosterone concentration for homeostasis. In contrast, in the maternally deprived group there is a significant positive correlation between corticosterone and body mass on PND 22 which may indicate the role visceral fat stores have in response to a nutritional stress at the time of weaning. Of particular importance is the fact that the increase in body mass and fat mass is not as a result of any external dietary modification and is already evident at an early stage of development. Moreover, in the maternally deprived animals our data show a positive relationship, between increasing body mass, associated with increased visceral fat stores, and the inflammatory markers, white cell count and plasma IL-6 concentrations.

Immunologically, the significant increase in white cell counts in the MD rat pups at weaning is indicative of an early inflammatory profile. Importantly, the increased WCC was significantly correlated with the increase in body mass and fat mass. Although there were no significant differences between the groups in IL-6 plasma concentration, on either the day of weaning or 24 hours later, there is a positive correlation on PND 22

between body mass and IL-6 in the maternally deprived group. Our data indicate that the relationship between the immune system and increased body mass is also detectable at an early developmental stage, namely weaning.

In order to approximate the stress associated with the disruption of human maternal attachment, our animals were exposed to a modified version of the standard maternal deprivation protocol commencing on PND 7 and extending to PND 21. Previous research has identified the specific period of PND 2 -14, in murine neonatal development, as the stress hypo-responsive period (SHRP), in which the pup requires good maternal care to modulate stress levels (Meaney, 2001). Stress during the SHRP period is known to prime the HPA axis and cause significant and detectable stress responses following a later life stressor (Faturi *et al.*, 2010; Gunnar & Donzella, 2002; Levine & Lewis, 1959; Levine *et al.*, 1992). However, a number of researchers have investigated the impact of maternal separation at different times during the neonatal period (Ellenbroek & Cools, 2002; Hancock *et al.*, 2005; Lehmann *et al.*, 2002; Macri *et al.*, 2011; van Oers *et al.*, 1997, 1998). In our variation of the maternal deprivation protocol extending into the third week of neonatal life, we have shown that the stress of forced weaning results in a significant difference in the corticosterone concentrations between the NMD and MD groups as early as PND 22. Heim *et al.* (2000) have proposed that a decreased HPA response should also be considered as an abnormal response to stress. The expectation that stress results in an increase in glucocorticoid secretion has been questioned, with significant research indicating the possibility of a bi-directional HPA axis response viz. hypocortisolaemia versus hypercortisolaemia

(Gustafsson *et al.*, 2010; Heim *et al.*, 2000; Macri *et al.*, 2011; Pervanidou, 2008; Ward *et al.*, 2004; Yehuda & Seckl, 2011). Our protocol and the resultant data provide evidence for early detection of hypocortisolaemia which we suggest should also be considered when evaluating early life stress responses (Heim *et al.*, 2000; Fries *et al.*, 2005; McEwen, 2006).

The suppression of the corticosterone stress response may be due either to adaptation to the separation protocol or due to the fact that the MD animals are significantly heavier than those in the NMD group at this stage of development (Zeeni *et al.*, 2012). Clinically, a recent study by Miller *et al.* (2013) demonstrated that children exposed to chronic stress who exhibited dampened salivary cortisol levels had increased BMI scores. Moreover, Yehuda and Seckl (2011) have suggested the possibility that a dampening of corticosterone secretion in response to a stressor may alter the relationship between the HPA axis and the immune system. In our model, the lack of significant differences in IL-6 concentration on PND 21 and 22 is in contrast to increasing research which links IL-6 to both psychopathology and obesity in adolescents and adults (Bugge *et al.*, 2012). The lack of significance with IL-6 responses at this early stage in development is likely due to the highly variable non-gaussian distribution of our IL-6 data. Alternatively, the later life finding of increased IL-6 concentrations may be related to changes during early development on other immune system markers such as white blood cell activity (Khanfer *et al.*, 2010). Maternal deprivation and increased IL-6 has been correlated in a later life research in animal models for the investigation of

inflammatory bowel disease (Desbonnet *et al.*, 2010; O'Malley *et al.*, 2011) and for changes in nociceptive behaviors (Burke *et al.*, 2013).

In clinical populations, an increase in fat mass associated with an increase in inflammatory markers such as IL-6, has been found in adult patients with depression (McIntyre *et al.*, 2007; Pace *et al.*, 2006; Shelton & Miller, 2010, 2011) and borderline personality disorder (Kahl *et al.*, 2005). Our data show an increased correlation between IL-6 and body mass on PND 22 in the pups that experienced the maternal deprivation protocol. These findings suggest that IL-6 cytokine responses are associated with increases in fat mass, adding support for Shelton and Miller's (2010) hypothesis that a relationship exists with distress in early life, macronutrient accumulation in adipocytes and the increased secretion of IL-6 from adipocytes and adjacent macrophages.

Notwithstanding the lack of IL-6 significance in our study, we have found a significant increase in white cell counts in the maternally deprived neonate rat pups on the day of weaning. The white cell count of the non-maternally deprived rat pups is within the normal range for pups of four weeks of age (Petterino & Argentino-Storino, 2006). Maternal separation during the SHRP period of neonatal development has been shown to increase colonic permeability, bacterial translocation and inflammatory responses in deprived rat pups (Barreau *et al.*, 2004). Of note, is the significant variance in the white cell counts of the maternally deprived rat pups associated with an increased body mass. In clinical populations, increased white blood cells have been associated with obesity (Benson *et al.*, 2008; Phelan *et al.*, 2011). Thus, it would seem that the association of

IL-6 and WBC counts with body mass may be important diagnostically in children who have experienced early life stress, especially since increased weight gain in childhood may predispose an individual to later life metabolic syndrome (Kerkhof *et al.*, 2012).

Our protocol which included maternal deprivation into the third week of neonatal life may account for the inflammatory results in this study. Moreover, it is possible that once the maturing pups are mobile, in the third week of life, they are able to forage for pellet chips on the cage floor. We would suggest that the ingestion of normal rat chow at an earlier age may further alter the intestinal microbiome and exacerbate the previously noted changes to the brain-gut axis that occur in maternally deprived rat pups (Gareau *et al.*, 2006; O'Mahony *et al.*, 2009). Changes in the immune response to solid food may further prime the HPA axis, specifically the secretion of corticosterone and alter immune system responses. Furthermore, on PND 22, the positive relationship between body mass and corticosterone, IL-6 and WBC in the maternally deprived pups is evidence of an early allostatic load. We are in agreement with Juster *et al.* (2010) in proposing that the measuring of interactions between mediators and effects will allow for an earlier identification of individuals at risk. The etiological aspects of the growing obesity pandemic and later life metabolic syndrome need to be further clarified and we believe that our modified maternal deprivation protocol, into the third week of rodent neonatal life, may have exposed relevant data on obesity formation from early life. Without any manipulation in feeding schedules our data support the hypothesis that early life stress contributes to an increase in fat mass associated with differential corticosterone and IL-6 plasma concentrations (Boynton-Jarrett *et al.*, 2012; Jahng,

2011; Ryu *et al.*, 2008; Spencer & Tilbrook, 2011). Thus, good consistent maternal care during early life, known to be a significant component for the stable development of the stress modulation system (Fries *et al.*, 2005; Levine 1957; Raineki *et al.*, 2010), is also required for regulation of the immune system and possibly as a preventative measure against the development of obesity.

In summary, our model of maternal deprivation more closely approximates maternal care disruption experienced by human children in a working population of women (Belsky, 2001). Furthermore, the relationship between maternal deprivation and increased body mass, at this early stage of development, has not been previously described and merits further investigation. Specifically, there is a growing need to intervene during early development in order to ameliorate psychopathologies that have been linked to early life stress, immunopathology, obesity and the development of metabolic syndrome. We believe that it is the responses to extreme stress, either hypo-responsive or hyper-responsive, that hold the key to the variable psychiatric and somatic diagnoses that all appear to be related to early life stress in clinical populations. Moreover, any future assessment of HPA axis reactivity should also include a more extensive investigation of peripheral immune system markers in the etiology of stress disorders associated with early life stress. In the light of extensive research on the long term health implications of early life stress, we believe that to achieve clinical relevance for early treatment programs, the evaluation of peripheral biomarkers are an important research consideration. Thus, notwithstanding the concerns raised about the applicability of an animal model in understanding the development of psychopathology

and specifically attachment difficulties we believe that the present study is important as it provides a novel investigation into the relationship between HPA axis reactivity, immune system markers and body mass at an early stage of development.

Acknowledgments

We would like to thank all the staff of the Central Animal Service for assistance with surgery and care of the animals. In addition, we would like to thank our Honours students Melissa Kock and Aaisha Moodley for their contribution to the work on white blood cells and body mass. This work was supported by Faculty Research Grants from the University of the Witwatersrand.

CHAPTER 5

INCREASED VISCERAL OBESITY IN JUVENILE FEMALE SPRAGUE-DAWLEY RATS FOLLOWING CUMULATIVE LIFE STRESSORS: IMPLICATIONS FOR FUTURE RESEARCH.

Abstract

The diagnosis of psychiatric disease in adolescence is controversial due, in part, to significant developmental changes to promote growth and maturation at this age interacting with attempts to maintain stability in the stress response system following early life adversity. In adulthood, increasing evidence indicates that psychiatric disorders are associated with early life stress, hypothalamic-pituitary-adrenal (HPA) axis dysregulation, inflammatory processes and central obesity. Moreover, animal studies following early life stress have shown dysregulation of the HPA axis, the immune system and neurobiological changes in adult animals. Importantly, there is a growing acknowledgement that early diagnoses of psychiatric conditions in adolescence may improve the life-long prognoses for youth affected by early life stress. Thus, the current study aimed at investigating the impact of foot-shock stress during the juvenile developmental stage in rats previously exposed to neonatal maternal deprivation. We assessed the impact of foot-shock stress on circulating corticosterone, interleukin-6, white blood cell count, body mass and visceral fat mass in juvenile female Sprague Dawley rats. No significant differences were found in corticosterone or IL-6. However, there was a significant increase in visceral fat mass during the juvenile stage ($p < 0.0001$). Critically, this research has raised vital and intriguing questions regarding the type of stress experienced, moderating factors and other potential circulating biomarkers.

Introduction

Adolescence, the transition period between childhood and adulthood, is known to be difficult for many children (Ayer *et al.*, 2013; Eiland & Romeo, 2013; Goodyer *et al.*, 2014). Adversity and difficulties in adolescence are common, with nearly nine million children in the United States, who have experienced adverse childhoods (Eiland *et al.*, 2012). Difficulties in adolescence may occur following early life adversity when the adolescent is attempting to maintain stability in their stress response while, developmentally, growth and maturation is occurring associated with significant changes in hormonal systems, physical appearance, cognition and parent-peer relationships (Hastings *et al.*, 2011; Vandell *et al.*, 2010). Although behavioural indicators of psychological distress may be evident in adolescents, no definitive, objective and measurable biological indicators have been established (Ayer *et al.*, 2013; Miller & Cole, 2012). In particular, the psychiatric personality disorder, Borderline Personality Disorder (BPD), associated with early life stressors and sexual abuse may result in significant behavioural dysregulation during adolescence (Belsky *et al.*, 2012; Chanen *et al.*, 2008; Zanarini *et al.*, 1997).

However, the diagnosis of a psychiatric disorder based on behavioural symptoms (APA, 2013) is extremely difficult and controversial during adolescence, possibly due to the major developmental changes (Owens, *et al.*, 2014; Yen *et al.*, 2013). There is evidence that borderline related characteristics are evident in adolescents and are associated with an increased risk of a later diagnosis of BPD in adulthood (Belsky *et al.*, 2012). Importantly, traumatic early life experiences are known to alter both physiology and

neurobiology (Herman *et al.*, 1989) and to be a significant factor in the development of psychiatric disorders such as depression (Miller & Cole, 2012), anxiety and addictive disorders (Eiland & Romeo, 2013). Thus, it is becoming apparent that earlier identification and treatment of psychiatric disorders may moderate long term neurobiological sequelae (Belsky *et al.*, 2012; Schwartz & Bahn, 2008).

Stress during vulnerable periods of life is associated with endocrine and neurobiological changes (McEwen, 2006) and altered behavioral responses (Rentesi *et al.*, 2013) when individuals are exposed to later life stressors. In both humans and animal models, early life stressors have been shown to result in a dysregulated hypothalamic-pituitary-adrenal (HPA) axis as measured by glucocorticoid responses to a stressor (Levine *et al.*, 1992; McEwen, 2006; Veenama, 2009). The impact of early life stress in animals, such as differential rearing (Levine, 1957; Levine & Lewis 1959; Macri *et al.*, 2011; Meaney, 2001) and maternal deprivation and separation (Ladd *et al.*, 2004; Levine *et al.*, 1992) has been extensively investigated following later life stress in pre-clinical models. Although experimental models of early life adversity have found biological dysregulation in adulthood (Eiland *et al.*, 2012), there are few models that have tested the cumulative effect of life stressors on a subsequent stress response during puberty (Ayer, *et al.*, 2013; Daskalakis *et al.*, 2012). Furthermore, there is a growing body of work that indicates that there are individual differences associated with glucocorticoid secretion following early life stress (Heim *et al.*, 2000; Cutili *et al.*, 2010; Owens *et al.*, 2014). Evidence has noted that female adolescents with high risk factors for the development of psychiatric illness present with a higher salivary cortisol prior to the

development of a Major Depressive Disorder (MDD; Goodyer *et al.*, 2000). In contrast, in Dutch youth, Ayer *et al.* (2013) have shown that behavioural, emotional and cognitive dysfunction is associated with attenuated glucocorticoid secretion. They found an attenuated salivary cortisol secretion in response to a stressor associated with higher levels of behavioural dysregulation in Dutch adolescents who have previously experienced early life stress. Moreover, the dysregulation of the HPA axis has significant implications for the immune system with BPD patients demonstrating plasma interleukin-6 (IL-6) cytokine changes that are associated with HPA dysregulation (Kahl *et al.*, 2006). Miller and Cole (2012) have also shown that immunological changes are evident six months prior to a depressive episode in adolescents who have experienced early life adversity. Additionally, a key function of the glucocorticoids is their role in the mobilization of energy reserves to meet the metabolic requirements. Thus, in the light of the obesity pandemic, significant links have been hypothesized between early life stress, HPA axis and immune dysregulation, obesity and psychiatric disorders (McIntyre *et al.*, 2007; Shelton & Miller, 2011).

The increasing obesity epidemic in adolescents has raised questions regarding the role of visceral fat following in cumulative life stressors (Evans *et al.*, 2013). Central obesity highlights the relationship between early and later life stressors and HPA axis dysregulation. Shelton and Miller (2011) suggest that central adiposity is associated with increased inflammatory signals that result in the induction of depression. Moreover, in a murine model of early life and later life stress, neuroinflammatory mechanisms have been shown to reactivate behavioural symptoms of depressed mood

(Wohleb *et al.*, 2013). Additionally, plasma IL-6 is increased in obesity and appears to be related to an infiltration of macrophages to areas of fat deposits (Galic *et al.*, 2010). Thus there is a growing need to identify and treat at risk adolescents in order to ameliorate later life distress (Ayer *et al.*, 2013; Belsky *et al.*, 2012; Goodyer *et al.*, 2014; Owens, *et al.*, 2014; Pajer *et al.*, 2012).

Although there is a significant focus on the neurobiological impacts of life stress, a critical need exists to identify and validate peripheral biomarkers associated with the cumulative impact of early life stress in an animal model to enable further research with youth at risk of later life psychiatric disorders (Daskalakis *et al.*, 2012; Pajer *et al.*, 2012; Pryce *et al.*, 2005; Schmidt *et al.*, 2008). The present study investigated the impact of foot-shock stress on animals that had previously experienced maternal deprivation during the neonatal stage of development. We investigated the biomarkers corticosterone and IL-6, 24 hour faecal glucocorticoid metabolite (FGM) secretion, white blood cell (WBC) counts, body mass, fat mass and visceral fat mass.

Methods

Animals

Twenty-four Sprague-Dawley female rats and their offspring were bred in the University of Witwatersrand Central Animal Service. To achieve a pregnancy, females obtained from our in-house breeding program were placed with a male Sprague-Dawley rat for five days. The females were checked daily for a vaginal plug. In order to minimize any further ante-natal stress, as soon as a successful mating had occurred the dams were

group-housed (2-4 animals per cage) and remained undisturbed in a designated pre-natal area of the animal unit. The principal researcher was responsible for regular bi-weekly husbandry of the animals from this point. Three days prior to the expected delivery date the pregnant females were separated and placed in individual 47 x 25 x 21 cm^3 clear plastic cages. The nursery was maintained on a 12/12 h light/dark cycle with lights on at 06h00. Controlled ambient temperatures were 24^oC, with humidity at 55%. Standard rat chow and water were available ad libitum. The day of delivery was designated post-natal day (PND) 0. Post-delivery, each litter was housed with its own dam until weaning on PND 21. To avoid any confounding stressors, litters were neither culled nor cross-matched to different dams. All experimental procedures were approved by the University of the Witwatersrand Animal Ethics Committee (certificate 2005/74/6), consistent with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals revised 1996.

Experimental Design

Maternal deprivation. Maternal deprivation is a well validated stress procedure (O'Mahony *et al.*, 2009; Veenema, 2009). Our variation of the maternal deprivation protocol was implemented from PND 7 – 20. Dams were randomly assigned to either the maternal deprivation (MD) group or the non-maternally deprived (NMD) group. In order to approximate the stress associated with the disruption of human maternal attachment, our animals were exposed to a variation of the standard maternal deprivation protocol, commencing on PND 7 and extending to PND 21. Dams and pups were left undisturbed until post-natal day seven (PND 7) after which the regular bi-weekly litter

changing strategy was resumed by the principle investigator. From PND 7, dams from the MD group were separated from their pups for three hours daily between 09:00 – 12:00 each day and placed in a soundproof room ten metres away from their pups. In contrast, the control group, the non-maternally deprived (NMD) pups, remained undisturbed with their dam. Litters were weaned, sexed, weighed, and placed in same sex group housing on PND 21. At weaning, animals from the NMD and MD deprived groups were group housed with two or three per cage until PND 40.

Foot-shock: On day PND 42, the female juvenile rats from the NMD and MD groups (see Figure 1) were randomly assigned to either the foot-shock (FS) or the sham condition, non-foot-shock (NFS). Furthermore, to control for the possible effect of the novel experience in the sham condition, a second control group of NMD rats were left completely undisturbed except for bi-weekly litter changes and designated as the Naïve group. Rats that were randomly assigned to either FS or NFS (sham) condition were individually placed in a cage adapted to administer foot-shock.

In the foot-shock apparatus, a plexiglass box (28 x 22 x 27 cm) with a grid metallic floor of the box connected to a controller (H13-16 Precision Regulated Shocker, Colbourn Instruments, Pennsylvania, USA) rats were allowed an acclimation period of 2 minutes. In the third minute they experienced a mild intermittent foot-shock of 2 mA at 1.6 Hz of one second duration every 20 seconds. The FS procedure was conducted on PND 42, PND 44 and PND 47 (Louvart *et al.* 2005; Sacerdote *et al.* 1994, Shurin *et al.* 1995). In the sham condition (NFS), rats were placed in the foot-shock apparatus, but no shock

was administered. On PND 47, immediately following the third foot-shock presentation, each rat was weighed and anaesthetized for cardiac puncture blood collection.

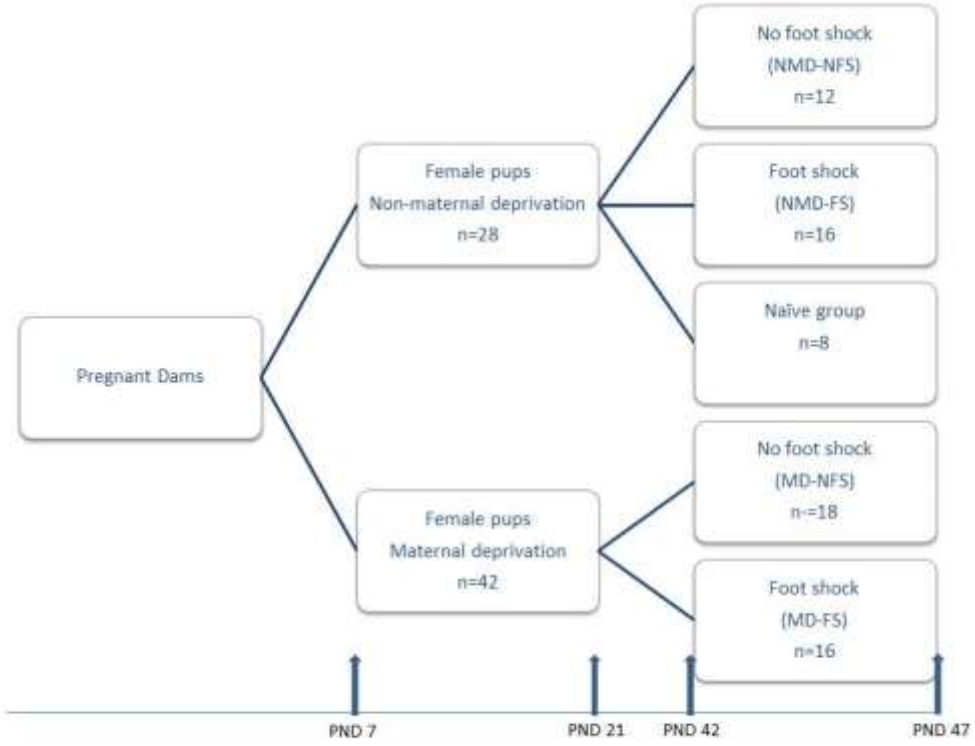


Figure 1. Experimental design.

Figure 1 provides an outline of the post-natal days of the protocol and the associated procedures.

Behavioural assessment. The behaviour of the animals in the foot-shock apparatus was observed and recorded before, during and after foot-shock. The number of behavioural responses occurring within the three minutes was recorded. Behavioural responses were recorded in the following categories: (1) Exploratory: which included rearing up the side of the cage and sniffing or (2) Response to foot-shock: which included freezing, attempts to escape, partially shut eyes and squealing on the next foot-shock presentation.

Experimental phases

Logistically, due to the large number of animals utilized in the current research project the experiments were divided into two phases. Importantly, by doing so we were able to investigate additional hypotheses by the serendipitous finding of an increased body mass in the maternally deprived animals that we had not anticipated in phase 1. Firstly, we investigated the cumulative impact of neonatal maternal deprivation and a juvenile foot-shock stress on the biomarkers corticosterone and IL-6. Secondly, in phase 2, we also assessed the maternal deprivation stress and foot-shock stress on 24 hour faecal glucocorticoid metabolite (FGM) secretion, white blood cell (WBC) counts, body mass, fat mass and visceral fat mass.

Phase 1.

In the first phase we examined the effects of three sessions of foot-shock stress on HPA axis activity and IL-6 in rats. As detailed in Figure 1, the MD and NMD groups were randomly assigned to either the foot-shock (FS) condition or the sham foot shock (NFS) condition. Furthermore, as noted the Naïve group were not exposed to the foot-shock apparatus. Thus, our groups in Phase 1 at PND 42 were NMD-NFS (n=10), NMD-FS (n=11), MD-NFS (n=10), MD-FS (n=10) and Naïve (n=8).

Phase 2.

Due to the lack of significance between the groups in Phase 1, the same experimental procedure was followed but additional investigations into 24 hour faecal glucocorticoid measures, white blood cell counts and visceral fat content were done. Moreover, given

the lack of significance of IL-6, we did not analyze IL-6 further. Thus, our total group sizes were different for phases 1 and 2.

Faecal collection. In phase 2, twenty-four hour faecal samples from all groups were collected for three days prior to the foot-shock protocol and every day during the protocol from PND 40 to PND 47 when the animal was killed. Cages were replaced with new clean sawdust bedding at 9h00 from PND 39 until PND 47. As the animals were group housed, body mass was taken each day to obtain the total weight of all animals in each cage. Faecal deposits for the last 24 hours, reflecting FGM output of the previous day, were removed from the sawdust bedding and stored at -70˚C until the extraction process.

Blood collection. Following the final foot-shock on PND 47, between 09:00 – 11:00, blood was collected by cardiac puncture for corticosterone and IL-6 determination from each of the groups. The Naïve animals were taken directly from their cage for weighing, anaethetization and cardiac puncture. Blood (2-3 mls) was obtained by cardiac puncture within four minutes of anesthesia, as per the recommendations of Arnold and Langhans, (2010). Following cardiac puncture animals were not revived but were killed using 200mg per kg intra-cardiac sodium pentobarbitone (Kyron Laboratories, Benrose, RSA). Blood was collected in a heparinized tube and centrifuged at 1000xg for 10 minutes at 4°C. Plasma was aliquoted and stored at -80°C for later determination of plasma corticosterone and cytokine concentrations.

Analyses

Behavioural assessment. Initially all rats explored the foot-shock apparatus. Exploratory behaviours, sniffing and rearing up on the side of the cage were scored. However, following the first foot-shock exposure, distress behaviours in the form of freezing, cage escape and partially closed eyes were noted in the animal that received the foot-shock but not the sham animals who continued with exploratory behaviours.

Plasma corticosterone determination. Plasma corticosterone concentrations were determined, on PND 47 following the final foot-shock, sham procedure and on the Naïve group. A rat double-antibody 125I-corticosterone radioimmunoassay kit (MP Biomedicals, Orangeburg, NY) was used as per manufacturer's instructions. The lower detection limit for the corticosterone kit was 7.7 ng/ml. Inter-assay variability for the assay was less than 6.9% while intra-assay variation was less than 7.3%.

Extraction of faecal glucocorticoid metabolites (FGM). Glucocorticoid metabolites in rodent faeces collected over each 24 hour period from PND 40 -47 (see Table 1) were used to assess HPA axis activity. On the day of analysis, faecal samples were left to thaw for one hour before being finely homogenised. A well-mixed sample of approximately 0.1grams is used to extract the metabolites in an 80% methanol solution. The solution is placed on a rotational mixer for 24 hours. Thereafter the samples were centrifuged at 3000rmp for 10minutes at 4°C and the methanol supernant was extracted and stored at -20°C until the radio-immunoassay for FGM was done.

FGM determination. Importantly, as the animals were group housed, FGM was determined as a percentage of body mass of the animals in the cage. The use of RIA to detect FGM in faeces has been validated by Harper and Austad (2000). A rat double-antibody 125I-corticosterone radioimmunoassay kit (MP Biomedicals, Orangeburg, NY) was used as per manufacturer's instructions. The lower detection limit for the corticosterone kit was 7.7 ng/ml. Inter-assay variability for the assay was less than 6.9% while intra-assay variation was less than 7.3%.

IL-6 cytokine analysis. Plasma IL-6 cytokine concentrations for all the animals were determined using the BioSource Rat IL-6 Elisa (BMS625, Bender Medsystems, Austria). The lower detection limit for the kit was 8 pg/ml. Inter-assay variability for the assay was less than 10% while intra-assay variability was less than 5%.

WBC counts. White blood cell counts on PND 47 were evaluated manually by diluting 40µl of whole blood with 360µl Turks solution (1ml Gentian violet 1% aqueous solution and 2ml glacial acetic acid in 100 ml distilled water) to haemolyse red blood cells and increase visualization of the leukocytes. The diluted blood solution was added to a Neubauer Improved hemocytometer chamber (Electron Microscopy Sciences, Set Point Instruments, South Africa) and incubated for three minutes at room temperature prior to counting the WBC's using a 10x objective on the microscope.

Body mass and fat mass determination. Body weight (g) was measured for all animals prior to anaesthetization. Visceral fat, including perirenal deposits, were

determined for the phase 2 cohort of pups following the foot-shock procedure. Visceral fat was dissected out and weighed (Gerbaix *et al.,* 2010). Fat mass was expressed as a percentage of body weight.

Statistical Analysis

All data were analyzed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). Normality was tested with the D'Agostino and Pearson omnibus test. In data that were normally distributed we analyzed the groups using ANOVA with the Newman-Keuls Multiple Comparison Test. These data are presented as (means ± SD). However, data that did not conform to a normal distribution curve and were analyzed using a non-parametric Kruskal-Wallis ANOVA and subjected to the Dunn's post hoc comparison. Non-parametric data are presented as medians. Statistical significance was accepted at $p<0.05$.

Results

Behavioural response to foot-shock

The response of 90% of the rats to the first foot-shock (regardless of whether the rats had experienced maternal deprivation or not) was freezing, while 10% tried to escape from the cage. On the subsequent foot-shock sessions (PND 44 and PND 47) all the previously shocked animals froze immediately upon being placed in the cage and they remained frozen for the first two minutes in the apparatus, apparently in anticipation of being shocked. In the third minute when foot shocks were administered, 71% of the

animals tried to escape from the cage. Additionally, 100% of the rats squealed when shocked.

Plasma corticosterone

Figure 2 shows the plasma corticosterone concentration from all animals in phase 1 and 2 after the last foot shock procedure. There are no significant differences between the groups ($F(4,69) = 1.094$, $p = 0.3665$).

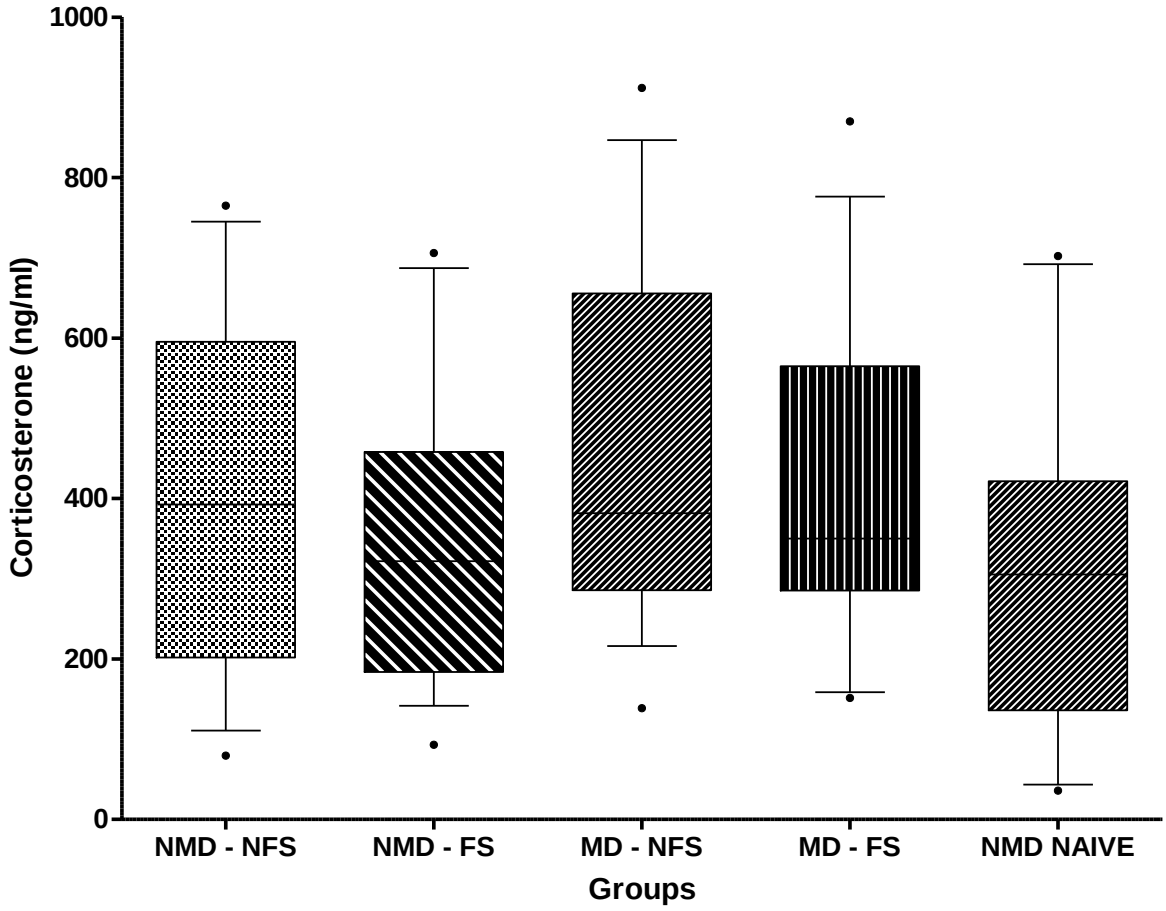


Figure 2. Plasma corticosterone concentrations

Plasma corticosterone concentrations, presented as a box and whiskers plots (10-90 quartiles), for all groups immediately following the final foot-shock on PND47. The groups are as follows: non-maternally deprived, no foot-shock (NMD-NFS; n=12), non-maternally deprived, foot-shock (NMD-FS; n=15), maternally deprived, no foot-shock (MD-NFS; n=18) and maternally deprived, foot-shock (MD-FS; n=15)

and Naïve (n=14). The upper and lower margins of the box plots indicate the 25th and 75th percentiles. The whiskers are drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers are shown as individual dots. The solid line within the box represents the median value.

Interleukin-6

Figure 3 shows the plasma IL-6 concentration on PND 47 for animals in both phase 1 and phase 2, following foot-shock. The data were not normally distributed. The medians between the groups were not significantly different (Kruskal-Wallis statistic = 5.557, p= 0.2347).

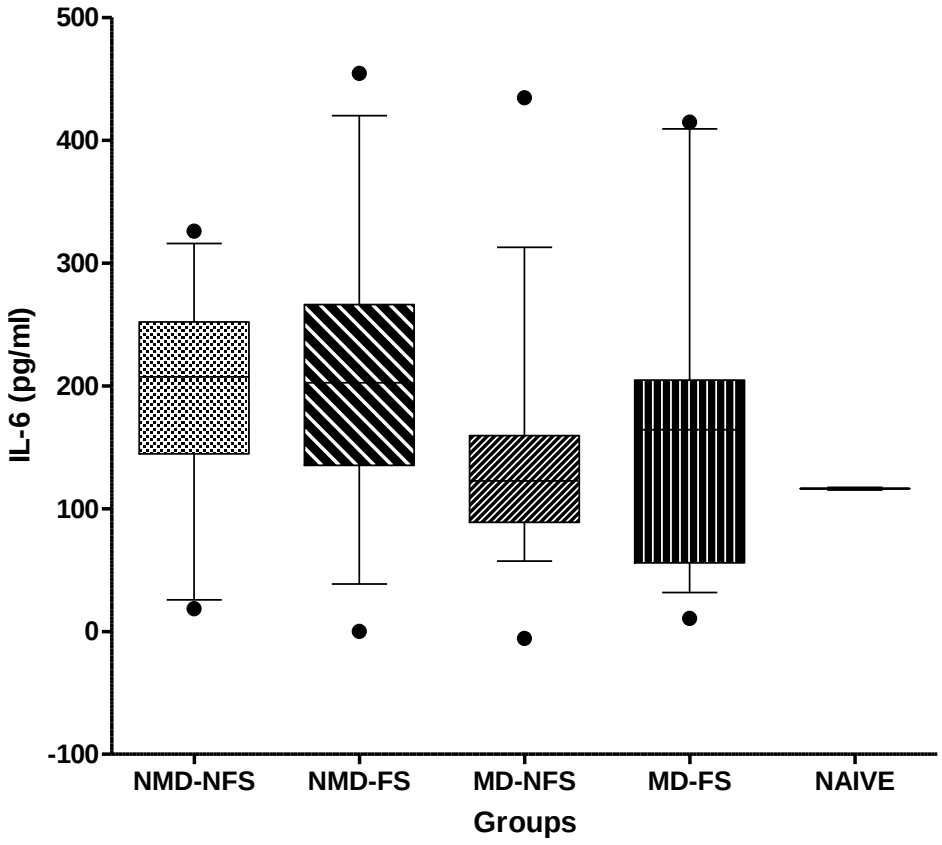


Figure 3. Plasma IL-6 concentrations

Plasma IL-6 concentrations, presented as a box and whiskers plots (10-90 quartiles), for all groups following final foot-shock (PND47). The groups are as follows: non-maternally deprived, no foot-shock (NMD-NFS; n=12), non-maternally deprived, foot-shock (NMD-FS; n=15), maternally deprived, no foot-

shock (MD-NFS; n=18) and maternally deprived, foot-shock (MD-FS; n=15) and Naïve (n=2). The upper and lower margins of the box plots indicate the 25th and 75th percentiles. The whiskers are drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers are shown as individual dots. The solid line within the box represents the median value.

Body Mass

Figure 4 shows the body mass following foot-shock and measured prior to anaesthesia, for all animals. ANOVA revealed significant differences between the between groups ($F(4,64) = 3.701$, $p = 0.0090$).

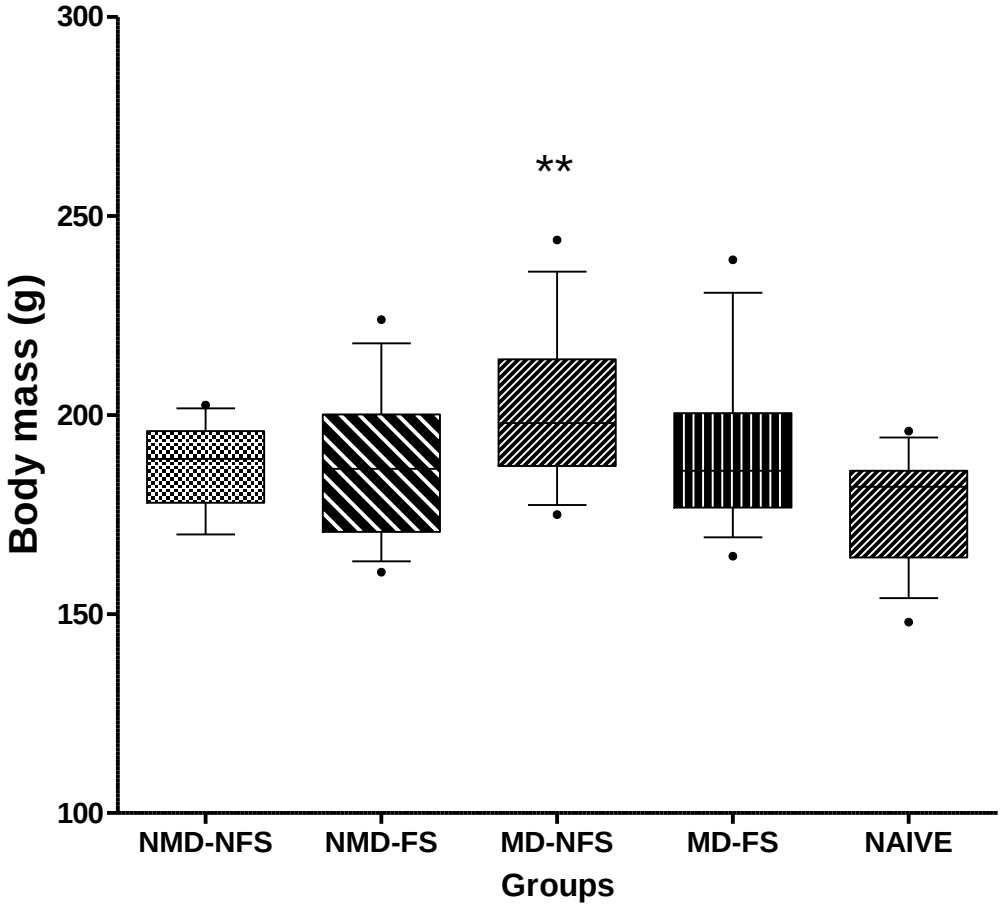


Figure 4. Body mass

Body mass, presented as box and whiskers plots for all groups following the final foot-shock (PND47). The groups are as follows: non-maternally deprived, no foot-shock (NMD-NFS; n=12), non-maternally

deprived, foot-shock (NMD-FS; n=15), maternally deprived, no foot-shock (MD-NFS; n=18) and maternally deprived, foot-shock (MD-FS; n=15) and Naïve (n=14). The upper and lower margins of the box plots indicate the 25th and 75th percentiles. The whiskers are drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers are shown as individual dots. The solid line within the box represents the median value.

** p< 0.005 Naive group vs MD-NFS group

Visceral fat mass

Figure 5 shows the comparison of visceral fat mass in the phase 2 animals. The data were not normally distributed, but there was a significant difference between the naïve group and both groups that had experienced maternal deprivation (Kruskal-Wallis statistic = 24.26, p< 0.0001).

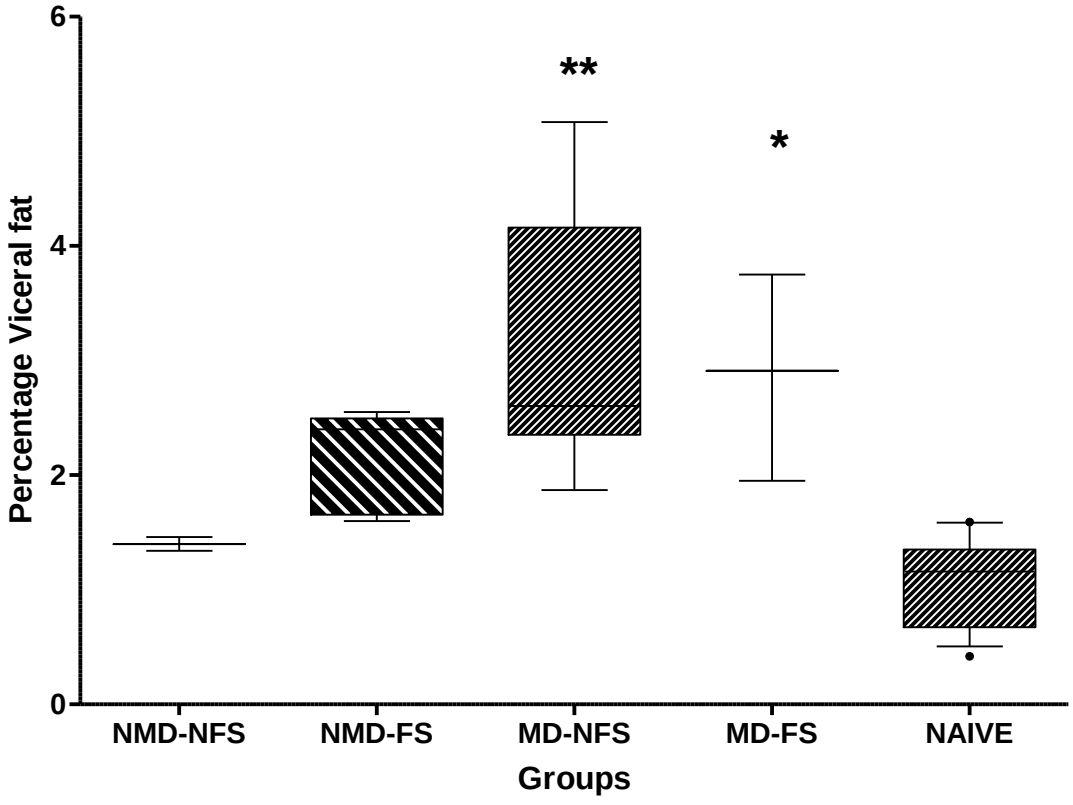


Figure 5. Percentage visceral fat

Percentage visceral fat presented as a box and whisker plots for all groups following final foot-shock (PND47). The groups were as follows: non-maternally deprived, no foot-shock (NMD-NFS; n=2), non-maternally deprived, foot-shock (NMD-FS; n=5), maternally deprived, no foot-shock (MD-NFS; n=8) and

maternally deprived, foot-shock (MD-FS; n=3) and Naïve (n=14). The upper and lower margins of the box plots indicate the 25th and 75th percentiles. The whiskers are drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers are shown as individual dots. The solid line within the box represents the median value.

*p<0.05 Naïve group vs MD-FS group

**p<0.005 Naïve group vs NMD-NFS group

White blood cell count

Figure 6 shows the comparison of white blood cell count measured in the Phase 2 animals, following foot-shock. The groups were not normally distributed. The Naïve group had significantly lower white blood cell counts than the NMD-NFS, NMD-FS and the MD-NFS animals (Kruskal-Wallis statistic =14; p= 0.0073).

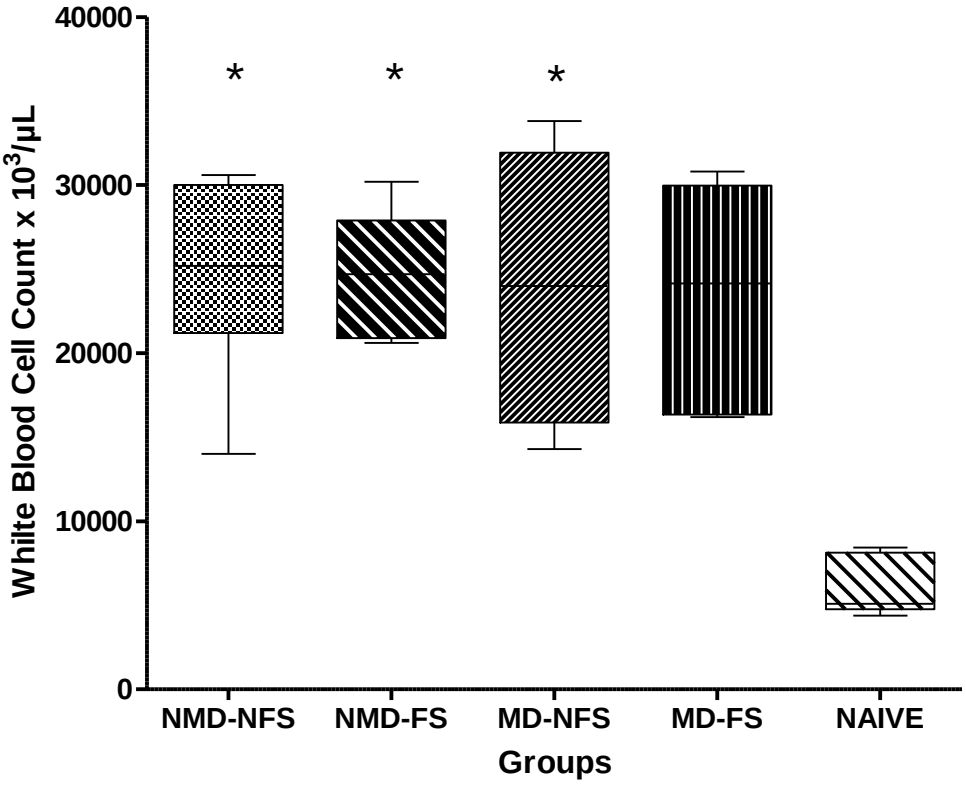


Figure 6. White blood cell counts

White blood cell counts presented as box and whisker plots (10-90 quartiles) for all groups following final foot-shock (PND47). The groups are as follows: NMD-NFS (n=7), NMD-FS (n=5), MD-NFS (n=6),

MD-FS (n=6) and Naïve (n=6). The upper and lower margins of the box plots indicate the 25th and 75th percentiles. The whiskers are drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers are shown as individual dots. The solid line within the box represents the median value.

*p<0.05 Naïve group vs NMD-NFS, NMD-FS, MD-FS groups

Faecal glucocorticoids

Figure 7 shows the comparison of faecal glucocorticoids, of the group housed animals, for 24 hours each day during the foot-shock period. A schedule documenting the PND, the processes and procedures and the associated data graph is presented in Table 1. FGM data from daily 24 hour faeces collection was used to determine a twenty-four hour measure of the effect of maternal deprivation and foot-shock on corticosterone secretion. These samples were used to determine and compare the effect of the neonatal stressor, maternal deprivation, on the subsequent response to the juvenile stressor, foot-shock. No statistical significance was found between any of the groups. However, there was evidence of a trend of decreased FGM values for the maternally deprived animals prior to, and during the foot-shock period. Additionally, all animals appear to habituate to the cage changes, sham novelty experience in the foot-shock apparatus and to foot-shock.

PND	Procedure	Timing	Stressor	Figure 7 - Graph
39	Cages changed and rats weighed at 09:00	Sawdust not analysed	Induction of cage switch stress	No data
40	FGM analysis of stressor - cage switch and new bedding 24 hours previously	Cage switch faecal sample	Cage switch stress	A
41	FGM analysis of stressor - cage switch and new bedding on PND 40	Cage switch faecal sample	Cage switch	B
42	FGM analysis of stressor - cage switch and new bedding on PND 40	Cage switch faecal sample	First foot shock presentation	C
43	FGM analysis of stressor - cage switch and first foot-shock presentation 24 hours previously	24 hours post first foot-shock faecal sample	Animals remain in their cages	D
44	FGM analysis of stressor - cage switch and first foot-shock presentation 48 hours previously	48 hours post first foot-shock faecal sample	Second foot shock presentation	E
45	FGM analysis of stressor - cage switch and second foot-shock presentation 24 hours previously	24 hours post second foot-shock faecal sample	Animals remain in their cages	F
46	FGM analysis of stressor - cage switch and second foot-shock presentation 48 hours previously	48 hours post second foot-shock faecal sample	Animals remain in their cages	G
47	FGM analysis of stressor - cage switch and second foot-shock presentation 72 hours previously	72 hours post second foot-shock faecal sample	Third and final foot-shock – animals killed	H

Table 1 Daily changes in FGM secretion

Daily changes in FGM secretion following cage switches and foot-shock. Graphical representation of FGM changes for all groups are presented in Figure 7.

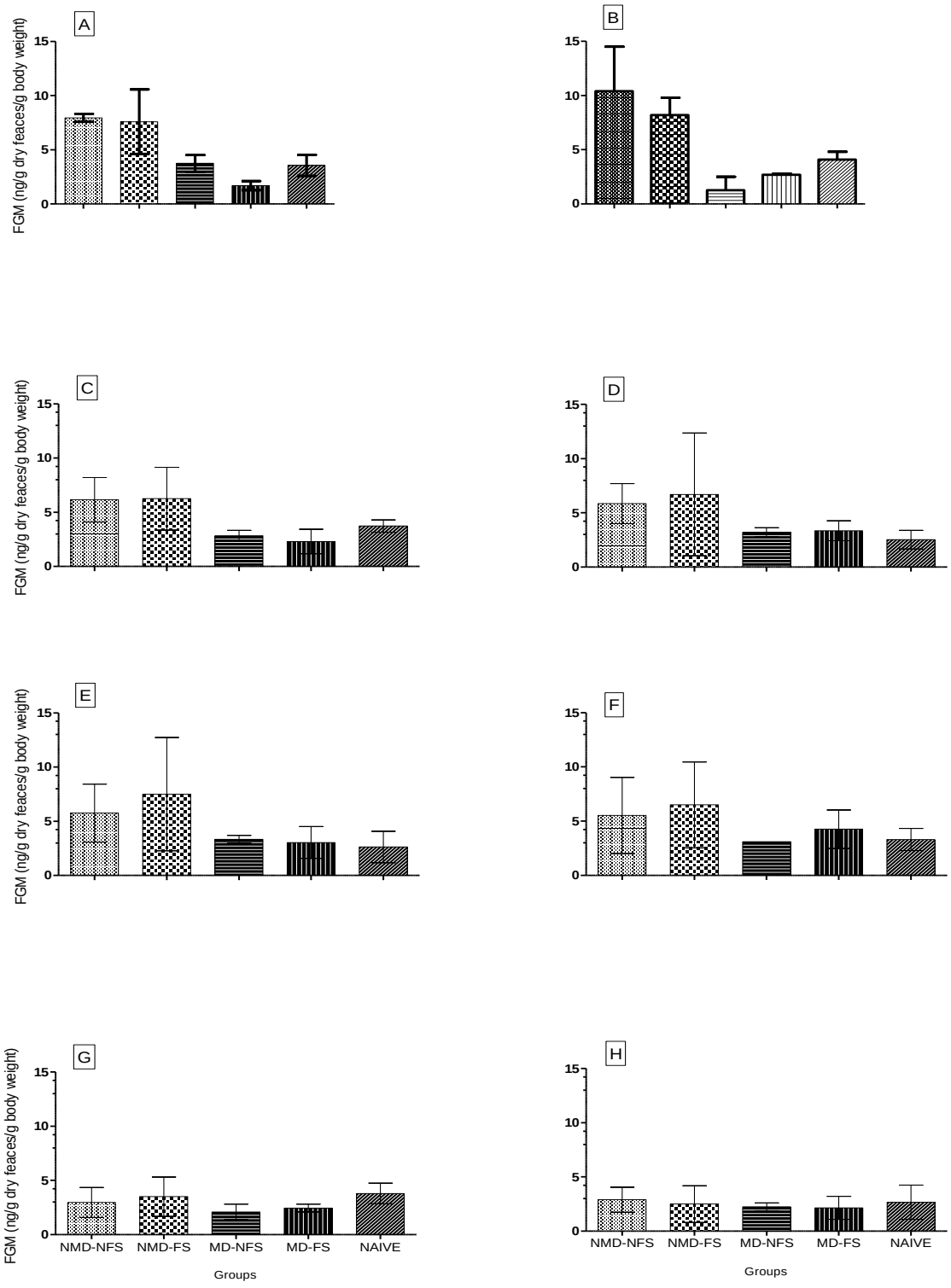


Figure 7. Corticosterone metabolite concentrations in faecal samples

Corticosterone metabolite concentrations in faecal samples (ng 0.05 g–1 faeces) from PND 40-47, before and during the foot-shock protocol for all 5 groups. Animals were group housed with two animals per

cages. The caged groups are as follows: NMD-NFS (n=4), NMD-FS (n=2), MD-NFS (n=2), MD-FS (n=2) and Naïve (n=4). Graph A reflects FGM secretion for the 72 hours prior to the first foot-shock on PND 42. Graph B reflects FGM secretion for the 48 hours prior to the first foot-shock on PND 42. Graph C reflects FGM secretion for the 24 hours prior to the first foot-shock on PND 42. Graph D represents FGM secretion for the first-24 hours post the first foot-shock presentation. Graph E shows the FGM concentration, 48 hours post foot-shock and following a non-foot shock day on PND 44, prior to the second foot-shock presentation. Graph F shows the FGM concentration on PND 45, twenty-four hours post the second foot-shock presentation. Graph G represents the FGM concentration 48 hours post the second foot-shock presentation on PND 46. Graph H represents the FGM concentration 72 hours post the second foot-shock presentation on PND 47.

Discussion

Although neurobiological changes are noted in adulthood following early life stressors, to date, there are no definitive diagnostic circulating biomarkers in the juvenile stage of development. The lack of identifying biomarkers in adolescence impedes the identification of risk of future psychiatric disorders which have been associated with early life stress. Thus, our goal in this research was to explore, in an animal model, whether the cumulative impact of early life stress was detectable in circulating plasma biomarkers during the juvenile stage of development. While clear behavioural differences in the foot-shock apparatus were evident in the animals that experienced the foot-shock protocol when compared to the sham groups, we found no significant difference in the plasma biomarkers corticosterone and IL-6. The lack of significant differences in plasma biomarkers corticosterone and IL-6 raised questions regarding a number of moderating factors. Firstly, sampling of blood at a single time point following the third foot-shock may have resulted in measuring an adapted corticosterone response to the relatively mild foot-shock protocol. Secondly, the novelty setting for the sham groups may also have increased corticosterone reactivity. Thus, in Phase 2 we

extended our investigation by analysing faecal glucocorticoid metabolites. Although, due to the small sample size and hence not statistically definitive, FGM revealed a trend of attenuated faecal glucocorticoids in the maternally deprived animals prior to foot-shock and following both the first and second foot-shock episodes. Importantly, this finding indicates that the animals that had experienced maternal deprivation had an attenuated 24 hourly corticosterone secretion in the juvenile period prior to the foot-shock protocol and did not respond to the cage changes, as expected, with an increase in FGM. Furthermore, following the foot-shock presentations, these animals maintained a blunted 24 hour FGM secretion. In contrast, the non-maternally deprived animals initially showed an increase in corticosterone secretion to the cage changes and the initial foot-shock procedure with attenuation of their stress response occurring only following the second foot-shock.

However, a significant finding in Phase 1 was the increase in body mass in the MD-NFS group exposed to our maternal deprivation protocol. We have previously shown that a variation of the standard maternal deprivation stressor from PND 7 - 21 is associated with corticosterone, IL-6 and white cell counts which are positively correlated with body mass and percent visceral fat mass in neonate animals (see Chapter 4, Results and Discussion). Previous research has identified a specific period from PND 2 to PND 14 as the stress hypo-responsive period (SHRP), in rodent neonatal development. Good maternal care modulates stress levels during the SHRP and is associated with low plasma corticosterone responses to stress in rat pups (Meaney, 2001). In pre-clinical trials, stress during the SHRP period is known to prime the HPA axis and cause

significant and detectable stress responses in adult animals following a later life stressor (Faturi *et al.*, 2010; Gunnar & Donzella, 2002; Levine & Lewis, 1959; Levine *et al.* 1992). Clinically, in human neonates good maternal care is also considered to provide a buffer for the development of a stable HPA axis (Gunnar & Donzella, 2002; Tang *et al.*, 2013). Controversially, the use of childcare facilities to allow mothers to return to work for economic reasons meant that children are left with non-familiar care-takers (Belsky, 2001). It is possible that this may have a negative impact on children which only becomes evident during adolescence (Vandell *et al.*, 2010). In order to address the possible impact of this early maternal loss our maternal deprivation protocol extended beyond the identified SHRP, into the period of corticosterone reactivity in the rat pup (Meaney, 2001).

Importantly, our modified maternal deprivation protocol, designed to evaluate the lack of maternal care from a later stage in infancy, reveals evidence of increase in body mass and visceral fat mass that is maintained into adolescence. Our result of increased body mass contradict the findings by Henningsen *et al.* (2012) of decreased body mass following a low maternal care stressor. However, the variation of the maternal care protocol that Henningsen *et al.* (2012) employed was a decrease in maternal care, as measured by licking and grooming behaviour. Their mild maternal stressor resulted in an anhedonic phenotype with low weight gain (Henningsen *et al.*, 2012). In contrast, Hancock *et a*l. (2005), note that in their variation of maternal care, foot-shock stress during the juvenile period of development induced binge eating in the offspring of low grooming dams.

The maternal deprivation stressor in our protocol results in both body mass and visceral fat mass increases in the juvenile stage of development. Increased visceral fat in humans is associated with early life stress and later life pathologies such as obesity, Metabolic Syndrome and Diabetes Mellitus Type II (Kahl *et al*., 2005; Rich-Edwards *et al*., 2010). The cumulative health risks associated with the growing obesity epidemic are of concern (Evans *et al.*, 2012; Ogden *et al.*, 2014). In particular, abdominal obesity and visceral fat have been shown to be associated with long term health implications (Evans *et al.*, 2012; Schröder *et al.*, 2014). An early predictor of lifelong obesity, in a national sample in the USA, appears to be increased body mass prior to kindergarten attendance (Gortmaker & Taveras, 2014). Importantly, although their data suggest that 47% of children who are obese in kindergarten have a risk of being obese when they are older, the authors are unable to differentiate the risk factors for early obesity in the research (Cunningham *et al.*, 2014).

Obesity associated with increased visceral fat has also been found in patients diagnosed with BPD and retrospective recall of early life stress. (Roepke *et al.*, 2010). Evans *et al.* (2012) note that exposure to early life stress in childhood results in larger gains in BMI in adolescence. The authors note that it has become increasingly important to understand the mechanisms associated with increased body mass (Evans *et al.*, 2012). HPA axis and immune system dysregulation in response to a stressor and the associated increases in body mass during the juvenile period may be important markers to detect allostatic load (Jahng, 2011). Therefore, in our study, the evidence of increased body mass in the animals that were previously maternally deprived prompted further

investigation into body mass and associated visceral fat stores in Phase 2. In particular, glucocorticoids have been implicated in the development of central obesity (Francis *et al.*, 2013; Jahng, 2011; Ibrahim, 2009).

However, in the current study, the single time point evaluation of plasma corticosterone following a relatively mild foot-shock protocol did not reveal any differences between the groups. Dagyte *et al.* (2009) note that acute foot-shock stress is not evident in HPA axis response 24 hours post the procedure. Cumulative changes in the brain may only be evident following chronic and severe foot-shock stress such as daily shocks for a period of three weeks (Dagyte *et al.*, 2009). Moreover, Hueston *et al*. (2011) note that in previously unstressed rats, plasma corticosterone is increased following a series of acute stressors which included a foot-shock protocol of 20 shocks in a period of thirty minutes. Furthermore, Rabasa *et al*. (2011) have demonstrated partial adaptation of the HPA axis to once a day medium intensity daily foot-shocks for an hour each day for seven days. In their high intensity protocol, there was no evidence of blunting in corticosterone or ACTH. In contrast, Beiko *et al.* (2004) using a Morris Water Maze protocol have shown a marked reduction in plasma corticosterone levels following repeated exposure to the same stressor, indicative of habituation to the stressor.

Thus, in phase 2 of the present study, the assessment of diurnal FGM excretion has provided a more comprehensive assessment of HPA axis activity over a 24 hour period (Sheriff *et al*, 2010) in animals undergoing a prolonged period of a mild stressor. The FGM results highlight a trend in the maternally deprived animals of an attenuated 24

hour corticosterone profile prior to the foot-shock protocol. As suggested by Suarez-Roca *et al.* (2014), the lower FGM levels in the maternally deprived animal may be due to either (1) an exhaustion of the HPA axis response or (2) an adaptation to stress. Moreover, in our study, by the end of the foot-shock protocol all groups have an attenuated FGM excretion suggesting adaptation to the stressor. Counter-intuitively, an increase in body mass has been associated with a blunted cortisol response to stress in pre-school children who experienced chronic stress in a poor environment (Miller *et al.*, 2013). In a recent study on stress and eating in the absence of hunger, Francis *et al.* (2013) note that post stressor, blunted plasma cortisol responses were seen in a majority of children in the age range 8-9 years. Variations in maternal care have also been found to result in binge eating (Hancock *et al.*, 2005), anhedonia and a stress susceptible phenotype (Henningsen *et al.*, 2012). In our animal model there is a trend to indicate that maternal care may be significant in ameliorating the development of increased visceral fat.

Furthermore, increased visceral fat has been associated with inflammatory markers such as increased IL-6 and macrophages (Shelton & Miller, 2011). Although we have been unable to discern IL-6 changes in the present research there were significant changes in WBC counts which may portend the future increases in IL-6. Dhabhar *et al.* (2012) have shown that following an acute stressor, the glucocorticoids are required to redistribute immune cells from the blood into different tissues in the body. Moreover, as Raison and Miller (2013a, 2013b) caution, slight changes in inflammatory markers that occur during episodes of stress may not be detectable in the early stages of chronic

stress. However, there may be a progressive impact on neuroendocrinology in later development. Thus it is possible that the foot-shock protocol employed in the present study was relatively mild and thus did not fully discriminate between the sham condition and the actual foot-shock. Additionally, the sampling of the plasma following the final foot-shock resulted in a habituation response in all the groups. Consequently, the major results of the current study are the increase in visceral fat and the attenuation in faecal glucocorticoid metabolites.

Critically, phase 1 and 2 of the current project have generated intriguing questions about moderating factors that may have obscured both the corticosterone and immune response to the stressor, foot-shock. Moreover, the role of moderating factors in adolescent children and juvenile animal models has not been fully investigated (Vandell *et al.*, 2010). Maternal care is considered to be a significant factor in developing coping mechanisms for later life stress (Belsky, 2001; Tang *et al.*, 2013). Childhood maltreatment has also been shown to result in significantly lower cortisol and ACTH responses to the laboratory stressor, The Trier Social Stress Test (Carpenter *et al.*, 2007). Suarez-Roca *et al.* (2014) have found that an attenuated glucocortiocoid response is associated with a reduced adrenocorticotropic hormone (ACTH) response to chronic stress. Furthermore, other hormones and peptides may also be associated with a decreased physiological response to the foot-shock stressor. Recent research on a group of peptides called the orexins has noted that under stressful conditions, orexins regulate physiological, behavioural and hormonal responses to stress (Chen *et al.*, 2014). Importantly, orexins, hypothalamic peptides stimulated by the HPA axis hormone CRH,

have also been associated with increased appetitive behaviours (Chen *et al.*, 2014). Immobilization in the foot-shock appartatus has been associated with increased production of orexins and is increased in rats that had experienced a brief foot-shocks (Chen *et al.*, 2014). In our study, the behavioural evidence of immobilization in the foot-shock apparatus is indicative of the possibility that there may have an elevation in orexin production. Additionally, a number of other appetitive hormones may be affected by early life stress and HPA axis dysregulation and need to be considered in future work. One of the key appetitive hormones is leptin, which has been associated with obesity and with the secretion of the glucocorticoids (Benardi *et al.*, 2013: Roubos *et al.*, 2012).

Adolescence is also associated with significant developmentally related biological changes which may be impacted by the cumulative effects of earlier life stress (Ayer, *et al.*, 2013). Goodyer *et al.* (2000) have shown that post-pubertal adolescent females at risk for a major depressive episode present with hypersecretion of dehydroepiandrosterone (DHEA) in the morning at 08.00 (Goodyer *et al.*, 2000). The moderating effect of the sex steroids in adolescence needs to be more fully investigated (Goodyer *et al.*, 2012). Our results parallel those of Helmreich *et al.* (2005) who found no differences in corticosterone responses in their experiment of chronic exposure to foot-shock stress over a period of 14 days. However, as corticosterone has also been considered in the regulation of the hypothalamic-pituitary-thyroid (HPT) axis Helmreich *et al.* (2005) found a significant decrease in circulating thyroid hormones thyroxine (T4), and triiodothyronine (T3).

Additionally, our animals in this project were group housed which may have resulted in a degree of modulation of stress responses (Pyter *et al*., 2014). Pyter *et al.* (2014) have shown that social isolation in a murine model of wound healing results in impairment of healing regardless of the bacterial load. In recently married couples who had experienced early life stressors, support from family ameliorates some of the lasting effects of the abuse (Tasca *et al.*, 2013). The obvious limitation of the current study is the small sample size in some of the groups. We therefore cautiously draw conclusions about the effect of early life stress on the accumulation of visceral fat. However, although in some instances, sample sizes are small, we believe that the present study raises vital questions regarding the possibility that maternal deprivation primes the long-term development of central obesity. Furthermore, the possibility exists that visceral fat is the aetiological factor of increased inflammatory markers that are present in later life (Després, 2006). Shelton and Miller (2011) have noted that a primary source of inflammatory markers in depressed patients is adipose tissue.

Thus, given the increasing obesity epidemic it is essential that we investigate all possible aetiological components of increased central adiposity (Evans *et al.*, 2012). Although this work currently does not demonstrate significant links to the glucocorticoids or the immune system, there is evidence of the impact of a maternal deprivation protocol that extends into the third week of neonatal life. Importantly, the increasing mobility of the pups at this stage may have allowed them the opportunity to forage for food on the cage floor. Thus, the work in Phase 2 of our model of early life stress represents significant advances in our understanding of the impact of a maternal deprivation stress that extends

beyond the SHRP. In particular, this research has also highlighted the importance of moderating factors in preventing both corticosterone and immune system dysregulation. We believe that this work will be critical for our future understanding of the aetiology of obesity.

In conclusion, evidence of stress responses in circulating plasma biomarkers, to cumulative stress, are not well characterized in the juvenile phase of development. There remains a critical need to find viable and effective peripheral biomarkers in adolescence that portend the development of neurobiological sequelae following early life stress. Moreover, as noted, pharmacotherapy and psychotherapeutic treatment of dysregulation in the endocrine and immune systems needs to be instituted before neurobiological damage. Thus, the future design of our research is intended to address the numerous facets that we have identified as limitations in the current project. The development of an applicable animal model to study the complex cumulative effects of early life maternal deprivation and later life stress responses is vitally important.

Acknowledgments

I would like to acknowledge and thank our Stress Lab Honours students, Tanusha Dukhan, Melissa Kock and Aaisha Moodley, for collecting some of the data during the second phase of this project. Furthermore, I would like to thank the staff of the Central Animal Service for assistance with surgery and care of the animals. This work was supported by Faculty Research Grants from the University of the Witwatersrand.

CHAPTER 6

SUMMARY OF THE CRITICAL HUMAN AND ANIMAL FINDINGS FOLLOWING EARLY LIFE STRESS.

Alterations in circulating cortisol and IL-6 concentrations in response to stress have been noted in adult patients diagnosed with psychiatric disorders. Importantly, dysregulated cortisol and immune system responses to stress are also noted in patients diagnosed with BPD who retrospectively report early life stress and sexual abuse (Kahl *et al*., 2006). Therefore the initial focus of the thesis was to investigate whether dysregulation of the peripheral biomarkers, cortisol and IL-6, were detectable in female children who had been sexually abused. Sexual abuse, associated with other early life stressors, results in a markedly different stress response to the forensic examination in some of the children. This finding merited further attention. Given the evidence of early maternal loss in some of the children, the aetiological implications of maternal deprivation on circulating corticosterone and IL-6 in a rat model were investigated. Moreover, as adolescence is considered a difficult time for children who have experienced early life stressors, the search for relevant circulating biomarkers was extended in the rat model into the juvenile stage of development.

Thus, as retrospective reporting of early life stress has been documented in adult patients and neurobiological changes have been identified in animal models following early life stress, this thesis was designed to answer the question: "Is it possible to identify dysregulation in peripheral biomarkers in early development following a severe stressor prior to the development of later life sequelae?" The results obtained from this research are summarized below in the context of the literature.

Sexual abuse, HPA axis and immune system dysregulation

In order to prevent the long-term effects noted in adult psychiatric patients who retrospectively report sexual abuse, there is a crucial need to evaluate whether physiological changes are evident when children first present at sexual abuse clinics. Importantly, to my knowledge, this is the first research to show evidence, in the children presenting to a forensic abuse clinic, of a dysregulated cortisol response to the stress of the forensic examination. As noted by Kudielka *et al*. (2004) stress reactivity in response a stressor is comparable, regardless of the time of day when the test is performed. This further supports our finding that some children failed to mount a stress response since they showed a cortisol level below 200 nmol/l in response to the standardized forensic examination. The study established that the cortisol response to the stress of the forensic examination was dependent on whether the children had experienced significant other life stressors. In Chapter 2, evidence is presented to show that children resident in a children's home exhibited an attenuated cortisol response in response to the forensic examination. However, a key consideration was whether this blunted stress response was actually correlated with being in the children's home or with another variable, possibly the loss of maternal care or other life stressors such as abuse in the family home. Moreover, the data in Chapter 2 represented a small pilot study which lacked a control group.

Finding a direct control group in this age group was difficult due to the fact that the stressor, the forensic examination was a specific stressor that could only be administered to the children presenting to the sexual abuse clinic with suspected sexual abuse.

However, in the follow up study (Chapter 3) it was possible to recruit a further number of children presenting to the sexual abuse clinic and to match them to a control group of children who were undergoing a bone density scan with associated blood and urine tests. Vitally important in this second study was that we were able to replicate the attenuated cortisol responses noted in our earlier pilot study. Children whose parents reported other early life stressors, such as death of a mother, paternal abuse or emotional abuse, also showed significantly attenuated responses to the stress of the forensic examination. One of the considerations from these results is whether the attenuation of the stress response may be due to these children having a different circadian rhythm at the time of the forensic examination. However, all investigations occurred during the morning between 10am to 12.30pm. Furthermore, as McEwen (2006) notes, regardless of the circadian rhythm, a stressor is expected to result in an increase in the stress response.

Crucially, in the non-stressed group of children who were involved in a novel event, it is evident that morning cortisol is in a far narrower range, namely 225.5 ± 47.5 nmol/l. As noted, although no direct comparison between the novelty of the DXA scan and the experience of the forensic examination was possible both groups experienced the novelty of attending either the hospital or the university and a potential stressor in the form of the blood draw. Using Vining and McGinley's (1987) formulae, we have been able to show that the children experiencing the DXA scan and subsequent blood draw show similar responses to those children experiencing the novelty of a mock MRI scans (Corbett *et al*., 2008).

Implicit in these results is the concern that under extreme stress, such as a medical or surgical trauma, there may be a significant disparity in a child's cortisol response. A dysregulated stress response in a medical or surgical emergency may have implications for the health of a child. This work also provides further support for the growing realization that attenuated cortisol responses may be a critical factor in the development of later life psychopathology (Ayer *et al.*, 2013; Cutuli *et al.*, 2010; Heim *et al.*, 2000) possibly due to the regulatory role that cortisol maintains in relation to the immune system. Moreover, with respect to cortisol and the stress response it is evident from this research that there are two significantly different groups of children who present to the forensic child abuse clinic, namely, children who respond to the stressor with an increase in circulating plasma cortisol and those who present with attenuation of their cortisol response. The other key consideration regards the impact that dysregulated cortisol secretion may have, both during and following a stressor, on the immune system. In the pilot study (Chapter 2) children with blunted responses to the forensic examination stressor had increased IL-6 levels. Following a motor vehicle accident, Pervanidou (2008) found that children with increased IL-6 levels in the morning were more likely to be given a diagnosis of PTSD six months later. Thus, there is evidence of physiological perturbations in cortisol and IL-6 in response to a stressor in the children from the sexual abuse clinic, which may portend the development of future psychopathology.

Another important finding of the research undertaken with the children from the Teddy Bear Clinic was that the attenuated responses to the stressor were associated with maternal loss or significant other life stressors in the family home. Tang *et al.* (2013)

note the multi-faceted role of the mother in ameliorating stress for a child. Clearly, in the children from the Teddy Bear Clinic, the role of the mother appears to be critical in enabling the child to respond appropriately to a stressor. Thus, a significant confounding variable to be considered is whether the physiology of these children had been impacted by a previous lack of maternal care. However, investigating the role of the mother in human children is associated with significant confounding variables. Due to the complexity of evaluating maternal care and the subjective nature of parental reports, a multi-modal approach to understanding attachment distress is required (Dewitte *et al.*, 2010). Consequently, as part of this thesis, a developmental rodent model was designed to evaluate the role of inadequate maternal care and the impact of a later life stressor on the physiological circulating concentrations of corticosterone (the rodent equivalent of human cortisol) and IL-6.

Visceral obesity associated with maternal deprivation and HPA axis and immune system dysregulation

Work with sexually abused children is complex, specifically when attempting to evaluate maternal care. Thus, having established that significant cortisol and IL-6 changes occur in the children from the forensic clinic, the impact of early life stress on plasma corticosterone concentrations and IL-6 was systematically investigated at two specific developmental stages in a pre-clinical rat model. Although extensive research in animal models has demonstrated that early life stress results in long term neurobiological sequelae (Holscher, 1999; McEwen, 2000; Rinne *et al.*, 2003), such neurobiological investigations are not applicable for everyday clinical situations in an abuse clinic in a low income economy. Furthermore, little clinical evidence exists of

early peripheral changes in circulating biomarkers that may be used to assist with diagnosis or the development of treatment protocols. A long-term developmental model would be useful in understanding some of the physiological changes that may occur in children prior to the progression to either psychiatric or somatic sequelae.

Thus, in order to evaluate maternal deprivation at a later stage in neonatal life, a variation of the standard maternal deprivation protocol was implemented. Circulating corticosterone and IL-6 levels were evaluated on PND 21 and, in a second cohort of animals, following the stress of weaning, on PND 22. On the day of weaning, although no significant differences were found between plasma corticosterone and IL-6, the pups which experienced maternal deprivation were significantly heavier. Therefore, I evaluated the impact of the stress of weaning and on PND 22, twenty-four hours later and found that there was a significant attenuation in plasma corticosterone in the maternally deprived group of pups. Serendipitously, the maternally deprived animals had a significantly increased body mass that was associated with an increase in visceral fat mass. Therefore, not only was it shown that it is possible to determine disparate stress responses at a very young age following maternal deprivation but that a significant increase in body mass is evident as a young age. The finding of an increase in visceral fat mass is significant as there are now indications that maternal deprivation may have implications for understanding the obesity epidemic (Evans *et al.,* 2012) and the consequent development of the metabolic syndrome (Després, 2006). Importantly, Jahng (2011) has noted that body mass changes are also indicators of early life stress and the development of an allostatic load. Exposure to early life stress may result in larger

gains in body mass index (BMI) in adolescence (Evans *et al.,* 2012). Moreover, as Belsky (2001) maintained, the early return to work by mothers and the subsequent placement in child care facilities was detrimental to infant well-being that may only become evident in adolescence.

Evidence of later life distress at juvenile stage of development

In adolescence there are behavioural indicators of psychological distress but there is a paucity of definitive, objective and measurable biological indicators which may assist in diagnosis of psychiatric disturbance (Ayer *et al*., 2013). Having established definitive evidence of a stress response to the forensic examination in girls following sexual abuse and in plasma corticosterone in the rat model following early life maternal deprivation, there is a need to evaluate possible long-term effects in a juvenile model of later life stress. In particular, the psychiatric personality disorder, Borderline Personality Disorder (BPD), associated with early life stressors and sexual abuse, has been shown to result in significant behavioural dysregulation during adolescence (Belsky *et al*., 2012; Zanarini *et al*., 1997). However, there is also significant evidence that differentiating psychiatric disorders in adolescent populations is fraught with confounding variables due to the developmental and maturational changes occurring at the same time (Hastings *et al.*, 2011; Vandell *et al.*, 2010).

Thus, we evaluated a foot-shock stressor in juvenile rats who had previously experienced the early life stressor, maternal deprivation. As evidenced (Chapter 5), no differences were found between any of the groups in the corticosterone and IL-6

responses to the stressor. It is possible that the impact of the relatively mild foot-shock protocol over a number of days allowed for a habituation to the stressor. Recently, it has been demonstrated that only a severe foot-shock stressor administered for longer periods elicits significant increases in corticosterone and IL-6 in plasma and brain regions (Dagyte *et al*., 2009; Hueston *et al*., 2011; Rabasa *et al*., 2011). In the light of the results it is evident that our low intensity foot-shock protocol was not sufficient to achieve significant differences in plasma levels using a single time point measure taken following the final foot-shock, of circulating biomarkers. However, a trend was found when 24 hour faecal glucocorticoid metabolites were analysed to evaluate stress responses before and during the foot-shock procedure. Prior to the foot-shock protocol, in animals which had experienced maternal deprivation, there was evidence of attenuated secretion of faecal glucocorticoid metabolites. Moreover, following the foot-shock protocol, the non-maternally deprived group also showed evidence of a trend for attenuation in the faecal glucocorticoid metabolites. As our sample size was small we cautiously draw conclusions, but it is clear from these results that using a 24 hour method of glucocorticoid evaluation requires more attention. Furthermore, it is important to note while attenuation of a stress response may indicate adaptation to the stressor it does not imply that there is no physiological cost to the animal (Rabase *et al*., 2011).

Thus, a key finding in the juvenile stressor research protocol was that body mass, associated with an increased visceral fat mass, was significantly increased in animals which had experienced maternal deprivation. Abdominal obesity, associated with later

life health implications, has been shown to be associated with increased body mass prior to kindergarten attendance (Gortmaker & Taveras, 2014). One of the functions of the glucocorticoids, to mobilize fat to storage sites closer to the liver, has been implicated in the development of central obesity (Francis *et al*., 2013; Ibrahim, 2009; Jahng, 2011). Thus, the increases in visceral fat mass during the juvenile period may be important markers to detect allostatic load (Jahng, 2011). Furthermore, the role of moderating factors in adolescent children and juvenile animal models needs to be more fully investigated. Although the small sample size, in the study with the juvenile animals, was a significant limitation there was evidence that visceral fat may be aetiologically associated with the long term sequelae from early life stress (Shelton & Miller, 2011).

In sum, the development of the psychiatric disorder BPD has been shown to be associated with difficulties with maternal attachments and with sexual abuse at a young age. Investigations into both sexual abuse and maternal care have revealed two key findings. Firstly, children that present at a sexual abuse clinic may be stratified into different clinical entities, namely hypocortisolaemia or hypercortisolaemia, based on their stress response to the forensic examination. Secondly, in an animal model of maternal deprivation, there is evidence of an increase in body mass and visceral fat mass following early life stress.

CHAPTER 7

CONCLUSIONS

Childhood resilience following early life stress is not a given. To date, a psycho-social framework has provided a comprehensive understanding of the behavioural and psychological ramifications following early life stress. However, burgeoning knowledge that early life stress is highly correlated with dysregulation of the stress response and the immune system and results in neurobiological changes indicates a clear imperative to establish whether this dysregulation is detectable in sexually abused female children prior to the development of long term psychiatric and somatic sequelae. Moreover, it is possible that symptoms related to damage that occurred early in life may only be evident as the individual matures and ages. Thus, there are two key findings from this research that need to be considered, namely attenuation of plasma glucocorticoids as a stress response and visceral obesity as a measure of allostatic load.

Attenuation of the stress response

Attenuation of cortisol response to a stressor is the key finding from the current research, occurring in both the human and the animal projects, following significant early life stress. A common misunderstanding about stress reactivity, found extensively in the literature and in popular media, that low cortisol responses indicate that stress is not being experienced by the organism may be false (Black & Garbutt, 2002; Heim *et al*., 2000; 2008; Nijm *et al*., 2007; Phillips *et al*., 2013). While a traditional view may have maintained that these children have habituated to their early life stressors and are thus resilient, the latest findings by Ayer *et al*. (2013) of attenuated stress responses occurring in youth with behavioural problems requires that further consideration be given to the impact that attenuation of circulating plasma cortisol as a stress response.

Increasingly in the last five years there has been significant research indicating that it is critical that the response to a stressful condition requires an increase in circulating cortisol to mobilize, contain and ameliorate the immune system response (Dhabhar *et al*., 2012: Raison & Miller, 2013). Importantly, this research has indicated that under conditions of severe early life stress there is an attenuation of the stress response. In the light of this finding there is a question about resilience. However, in contrast, other researchers have shown that hyper-responsiveness of the HPA axis is associated with depression and metabolic syndrome (Lamers *et al*., 2013). Importantly, some of these discrepancies may be accounted for by understanding which method was utilized to investigate cortisol output. In the present research, the single time point measure of glucocorticoid and immune system changes following a stressor did not allow for the investigation of a continued hypercortisolaemia following the stressor (McEwen, 2006). Thus, it is important to stratify the different stress response phenotypes that patients present with, namely increased HPA axis reactivity versus a dampening of the HPA axis response and to monitor these responses over the long-term.

Immunologically, although we found a significant inverse relationship in the children between the glucocorticoid cortisol and IL-6 in the pilot study, my research provided no clarity of the immune system changes that may facilitate the development of psychiatric disorders and somatic illness (Raison & Miller, 2013). Wohleb *et al*. (2013) have highlighted the link between chronic stress and the immune system. These authors have shown that under chronic stress conditions there is a movement of white blood cells from the spleen to the brain (Wohleb *et al*., 2013). It is possible that the impact of

inflammatory conditions, neuroinflammation, in the brain may be established early on in development when an individual first encounters chronic stressors.

Visceral obesity as a measure of allostatic load in young children and adolescents

In the maternal deprivation rat study, although no significant differences in IL-6 were found, there was evidence of a link between the immune system and the increased level of visceral fat in these animals. As noted, key difficulties occur when a response stress does not return to normal following a stressful event and results in a constant imbalance in the stress response, an allostatic load, for the individual (McEwen, 2006). Early life stress may be a key aetiological factor that is driving the current obesity epidemic. Importantly, some patients, abused as children and diagnosed with BPD in adult life, have an increase in both visceral fat and obesity (Frankenburg & Zanarini, 2006; Kahl *et al.*, 2005; Roepke *et al.*, 2010). Moreover, as noted, the adipocytes in visceral fat are recognised to secrete endocrine and immune system products (Galic *et al.*, 2010; Harwood, 2012; Shelton & Miller, 2010). Thus, our findings in the animal research protocols need to be verified in the young patients.

The future - towards a developmental trauma model

The current research has again highlighted the need for a developmental understanding of the impact of early life trauma (Ford *et al.*, 2013). Van der Kolk *et al*. (2005) petitioned the relevant DSM 5 reviewers for the introduction of a diagnosis for complex trauma, but were unsuccessful due to the lack of objective and definitive evidence. This thesis has begun to clarify what needs to be done to establish objective biological

evidence. Rinne *et al*. (2003) are focusing on the psychiatric effects of activation of the HPA axis in adult patients who have experienced early life sexual abuse associated with chronic post-traumatic stress disorder. Other researchers have found that maternal unavailability and deprivation in an infant's first year of life may result in activation and persistent hyper-responsiveness of the HPA axis (Rinne *et al.* 2003). Severe, prolonged or early emotional stress appears to impact the HPA axis resulting in a complex interwoven relationship between personality development, maturation, the endocrine system and the immune system.

Contradictory evidence exists regarding the type and levels of stress which may be deleterious to a child. A key factor emanating from this thesis is the need for a comprehensive longitudinal study of children at risk. Notwithstanding the difficulties of identifying a bio-signature in children who have been subjected to early life abuse, pursuing biomarker research with young children who have suffered abuse may allow us to delineate children at risk for psychopathology and will begin to elucidate potential long term implications for health in these children. In addition, this research has improved our understanding of the fundamental impact of HPA axis activation on the immune system messenger molecule, IL-6. In our study, early life stress followed by a stressful event provided clear evidence of attenuation in circulating cortisol levels. To address the limitations of the current research method of measuring a single immediate cortisol response to a stressor, it is imperative that on follow-up with these young patients a daytime salivary cortisol profile is done. It is also recommended that a cortisol awakening response and 24 hour urinary glucocorticoid metabolites be

evaluated. Furthermore, as Kapur *et al.* (2012) note, comparisons between normal and "psychiatrically ill" subjects may not be appropriate. Rather, to obtain clinical relevance we now need to concentrate on evaluating the differences between subgroups of patients with the same diagnosis.

There is also a pressing need for research into the interaction between other hormonal systems, genetic predisposition and specific environmental stressor to determine individual responses to HPA axis activation as well as the cellular mechanisms responsible for the apparent links between personality changes and chronic PTSD (Tarullo & Gunnar, 2006). However, as Tsao and Vasan (2013) note, the search for biomarkers needs to fulfil specific criteria that include ease of use and address the needs of patients with respect to improving diagnostic accuracy. Furthermore, treatment guidelines need to be established in the medical profession to enable medical personnel to adequately advise parents and care-givers on effective treatment strategies (Tsao & Vasa, 2013). Importantly, these considerations mean that some of these goals are currently unattainable. As stated at the outset of my thesis, we are only at the beginning of a long journey to discover ways of assisting the myriad children affected each day in every country in the world by life stressors that are beyond their physiological capacity to handle. Moreover, although the current thesis has focused on female children and therefore a female rat model, there is increasing evidence that the incidence of reported sexual abuse in young boys is a growing problem. In 2013 we re-evaluated the number of children attending the Teddy Bear Clinic and found that approximately 50% were boys between the ages of 6-12 years.

In sum, I have substantiated my contention that early life stress has direct and measurable biological impacts that are evident early in life. Therapeutically, further evaluation of endocrine and immune system biological markers as possible diagnostic markers for evaluating risk following early life stress is recommended. Moreover, there is a need to develop longitudinal studies to evaluate the impact of HPA and immune system responses to an early life stressor and later life. A better understanding of the impact of early life stress on biological parameters and the mechanisms associated with the development of lasting somatic and psychiatric pathologies may offer new possibilities for diagnosis, prognosis and treatment of children who have experienced an early life stressor. However, limitations with the research have to be acknowledged.

Vision, strategy and goals for future investigations into long-term consequences of early life stress

There is a growing imperative to understand the biological underpinnings of psychiatric disorders. The use of biomarkers to identify risk and to start preventive therapy is becoming an important consideration in many areas of medicine today. The ongoing biomarker discovery in psychiatric medicine and the nascent work towards establishing significant biomarkers that signal a possible prodromal stage of a psychiatric disorder are essential in helping to ameliorate future disease (Guest *et al.*, 2014; Schwarz & Bahn, 2008)). Since we recognise that sexual abuse is associated with later life psychiatric disorders and somatic disorders, there is increasing pressure to identify and ameliorate the impact of the stressor prior to the development of lifetime sequelae.

In conclusion, similarly to the wish expressed by Hanahan and Weinberg (2000, 2011), with their early treatise on the "Hallmarks of Cancer", the future vision that I argue for, is that the time is right for psychiatry and psychology to work towards the development of an integrated bio-psycho-social understanding of the impact of life stress on development. This thesis marks a beginning and contributes towards unravelling the complex relationship that exists between the individual, life stressors, the HPA axis and the immune system. Increasingly there is scientific proof that early life stress, especially in the form of sexual abuse, alters and dysregulates physiological parameters. It is important that the long-term impact of physiological disruption to both the stress and immune systems should be considered by every professional who deals with traumatized young children. A logical science that investigates biological changes and develops pertinent therapeutic interventions is now required to prevent the lasting impact of abuse.

CHAPTER 8

REFERENCES

Ainsworth, M.D. (1985). Attachments across the life span. Bulletin of the New York Academy of Medicine, 6, 792-812.

Akers, K.G., Nakazawa, M., Romeo, R.D., Connor, J.A., McEwen, B.S., & Tang, A.C. (2006). Early life modulators and predictors of adult synaptic plasticity. European Journal of Neuroscience, 24, 547-554.

Akirav, I., Kozenicky, M., Tal, D., Sandi, C., Venero, C., & Richter-Levin, G. (2004). A facilitative role for corticosterone in the acquisition of a spatial task under moderate stress. Learning and Memory, 11, 118-195.

Allen, A.P., Kennedy, P.J., Cryan, J.F., Dinan, T.G., & Clarke, G. (2014). Biological and psychological markers of stress in humans: Focus on the Trier Social Stress Test. Neuroscience and Biobehavioral Reviews, 38, 94-124.

Alvarez, P., Green, P.G., & Levine, J.D. (2013). Stress in the Adult Rat Exacerbates Muscle Pain Induced by Early-Life Stress. Biological Psychiatry, pii: S0006-3223(13)00339-9.

American Psychiatric Association. (2013). Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. Arlington, VA, American Psychiatric Association.

Anand, K.J.S., Ciskun, V., Thrivikraman, K.V., Nemeroff, C.B., & Plotsky, P.M. (1999). Long-term behavioural effects of repetitive pain in neonatal rat pups. Physiology and Behaviour, 66, 627-637.

Anisman, H. (2009). Cascading effects of stressors and inflammatory immune system activation: implications for major depressive disorder. Journal of Psychiatry Neuroscience, 34, 4-20. Retrieved from

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2612083/pdf/20090100s00002p4.pdf

Anisman, H., Hayley, S., Turrin, N. & Merali, Z. (2002). Cytokines as a stressor: implications for depressive illness. International Journal of Neuropsychopharmacology, 5, 357-373.

Anisman, H., Merali, Z., Poulter, M.O., & Hayley, S. (2005). Cytokines as a precipitant of depressive illness: animal and human studies. Current Pharmarmaceutical Design, 11, 963-972.

Anisman, H., Zaharia, M.D., Meaney, M.J., & Merali, Z. (1998). Do early life events permanently alter behavioral and hormonal responses to stressors? International Journal of Developmental Neuroscience, 16, 149-164.

Arnold, M., & Langhans, W. (2010). Effects of anesthesia and blood sampling techniques on plasma metabolites and corticosterone in the rat. Physiology and Behavior, 99, 592-598.

Ayer, L., Greaves-Lord, K., Althoff, R.R., Hudziak, J.J., Dieleman, G.C., Verhulst, F.C., & van der Ende, J. (2013). Blunted HPA axis response to stress is related to a persistent Dysregulation Profile in youth. Biological Psychology, 93, 343-351.

Badanes, L.S., Watamura, S.E., & Hankin, B.L. (2011). Hypocortisolism as a potential marker of allostatic load in children: associations with family risk and internalizing disorders. Developmental Psychopathology, 23, 881-896.

Barreau, F., Ferrier, L., Fioramonti, J., & Bueno, L. (2004). Neonatal maternal deprivation triggers long term alterations in colonic epithelial barrier and mucosal immunity in rats. Gut. 53, 501-506.

Bartolomucci, A. (2005). Resource loss and stress-related disease: Is there a link. Medical Science Monitor, 11, RA147-154.

Beiko, J., Lander, R., Hampson, E., Boon, F., & Cain, D.P. (2004). Contribution of sex differences in the acute stress response to sex differences in water maze performance in the rat. Behavioural Brain Research, 15**,** 239-253

Beitia, G., Garmendia, L., Azpiroz, A., Vegas, O., Brain, P.F., & Arregi, A. (2005). Time-dependent behavioral, neurochemical, and immune consequences of repeated experiences of social defeat stress in male mice and the ameliorative effects of fluoxetine. Brain, Behavior, and Immunity, 19, 530-539.

Belsky, D.W, Caspi, A., Arseneault, L., Bleidorn, W., Fonagy, P., Goodman, M., Houts, R., & Moffitt, T.E. (2012). Etiological features of borderline personality related characteristics in a birth cohort of 12-year-old children. Developmental Psychopathology, 24, 251–265.

Belsky, J. (2001). Emanuel Miller lecture developmental risks (still) associated with early child care. Journal of Child Psychology and Psychiatry, 42, 845-59.

Benes, F.M. (2010). Amygdalocortical circuitry in schizophrenia: from circuits to molecules. Neuropsychopharmacology, 35, 239-57.

Benson, S., Janssen, O.E., Hahn, S., Tan, S., Dietz, T., Mann K, … Elsenbruch S. (2008). Obesity, depression, and chronic low-grade inflammation in women with polycystic ovary syndrome. Brain, Behavior, and Immunity, 22, 177-84.

Bergink, V., Gibney, S.M., & Drexhage, H.A. (2013). Autoimmunity, inflammation and psychosis; A search for peripheral markers. Biological Psychiatry, http://dx.doi.org/10.1016/j.biopsych.2013.09.037

Berk, M. (2013). The DSM-5: hyperbole, hope or hypothesis. BMC Medicine, 11, 128.

Bernard, K., & Dozier, M. (2010). Examinationining infants' cortisol responses to laboratory tasks among children varying in attachment disorganization: stress reactivity or return to baseline? Developmental Psychology, 46, 1771-1778.

Bernardi, J.R., Ferreira, C.F., Senter, G., Krolow, R., de Aguiar, B.W., Portella, A.K., … Silveira, P.P. (2013). Early life stress interacts with the diet deficiency of omega-3 fatty acids during the life course increasing the metabolic vulnerability in adult rats. PLoS One. 8,e62031.

Binder, E.B., & Holsboer, F. (2012). Low Cortisol and Risk and Resilience to Stress-Related Psychiatric Disorders. Biological Psychiatry, 7, 282–283.

Black, P.H. (2003). The inflammatory response is an integral part of the stress response: Implications for atherosclerosis, insulin resistance, type II diabetes and metabolic syndrome X. Brain, Behavior, and Immunity, 17, 350-364.

Black, P.H., & Garbutt, L.D. (2002). Stress, inflammation and cardiovascular disease. Journal of Psychosomatic Research, 52, 1 – 23

Blalock, J.E., Bost, K.L., & Smith, E.M. (1985). Neuroendocrine peptide hormones and their receptors in the immune system. Production, processing and action. Journal of Neuroimmunology, 10, 31-40.

Booij, L., Wang, D., Lévesque, M.L., Tremblay, R.E., & Szyf, M. (2013). Looking beyond the DNA sequence: the relevance of DNA methylation processes for the stress-diathesis model of depression. Philosophical Transactions of the Royal Society B Biological Sciences, 368, 20120251.

Borghetti, P., Saleri, R., Mocchegiani, E., Corradi, A., & Martelli, P. (2009). Infection, immunity and the neuroendocrine response. Veterinary Immunology and Immunopathology, 130, 141-162.

Boscarino, J.A. (2004). Posttraumatic stress disorder and physical illness. Results from clinical and epidemiologic studies. Annals of the New York Academy of Sciences, 1032, 141-153.

Boschan, P.J. (2008). Childhood and trauma. The American Journal of Psychoanalysis, 2008, 68, 24–32).

Bowlby, J. (1952). *Maternal care and mental health.* World Health Organization, 2nd Ed. Retrieved from: http://whqlibdoc.who.int/monograph/WHO_MONO_2 (part1).pdf.

Bowlby, J. (1987 a). Attachment and Loss: Volume 1. Attachment. Reading: Cox and Wyman Ltd.

Bowlby, J. (1987 b). Attachment and Loss: Volume 2. Separation. Harmondsworth: Hazell Watson and Viney Ltd.

Bowlby, J. (1995) [1950]. Maternal Care and Mental Health. The master work series (2nd ed.). Northvale, NJ; London: Jason Aronson. [Geneva, World Health Organization, Monograph series no. 3].

Boynton-Jarrett, R., Rosenberg, L., Palmer, J.R., Boggs, D.A., & Wise, L.A. (2012). Child and adolescent abuse in relation to obesity in adulthood: the black women's health study. Pediatrics, 130, 1-9.

Breidenstine, A.S., Bailey, L.O., Zeanah, C.H., & Larrieu, J.A. (2011). Attachment and Trauma in Early Childhood: A Review. Journal of Child & Adolescent Trauma, 4, 274-290,

Bremner, J.D., Vythilingam, M., Vermetten, E., Southwick, S.M., McGlashan, T., Naseer, A., … Charney, D.S. (2003). MRI and PET study of deficits in hippocampal structure and function in women with childhood sexual abuse and posttraumatic stress disorder. American Journal of Psychiatry, 160, 924-932.

Briere, J., & Runtz, M. (1987). Post Sexual Abuse Trauma: Data and Implications for Clinical Practice. Journal of Interpersonal Violence, 2, 367 - 379.

Briere, J., & Runtz, M. (1988). Post Sexual Abuse Trauma. In G. E. Wyatt & G.J. Powell (Eds.), Lasting Effects of Child Sexual Abuse (pp. 85 - 99). California: Sage Publications.

Briere, J.N. (1992). Child abuse trauma. Theory and treatment of the lasting effects. Sage Publications Newbury Park.

Brown, G.R., & Spencer, K.A. (2013). Steroid hormones, stress and the adolescent brain: A comparative perspective. Neuroscience, 249, 115-128.

Buchanan, T.W., Tranel, D., & Kirschbaum C. (2009). Hippocampal damage abolishes the cortisol response to psychosocial stress in humans. Hormones and Behavior, 56, 44-50.

Bugge, A., El-Naanam, B., McMurray, R.G., Froberg, K., Nielsen, C.H., Muller, K., & Anderson, L.B. (2012). Sex differences in the association between level of childhood interleukin-6 and insulin resistance in adolescence. Experimental Diabetes Research, 859186. doi:10.1155/2012/859186, 1-6.

Burghy, C.A., Stodola, D.E., Ruttle, P.L., Molloy, E.K., Armstrong, J.M., Oler, J.A., … & Birn, R.M. (2012). Developmental pathways to amygdala-prefrontal function and internalizing symptoms in adolescence. Nature Neurosciences, 15, 1736-1741.

Burke, N.N., Llorente, R., Marco, E.M., Tong, K., Finn, D.P., Viveros, M.P., & Roche, M. (2013). Maternal Deprivation Is Associated With Sex-Dependent Alterations in Nociceptive Behavior and Neuroinflammatory Mediators in the Rat Following Peripheral Nerve Injury. The Journal of Pain, 14, 1173-1184.

Carpenter, L.L., Carvalho, J.P., Tyrka, A.R., Wier, L.M., Mello, A.F., Mello MF, … Price, L.H. (2007). Decreased adrenocorticotropic hormone and cortisol responses to stress in healthy adults reporting significant childhood maltreatment. Biological Psychiatry, 62, 1080-1087.

Carvalho Fernando, S., Beblo, T., Schlosser, N., Terfehr, K., Otte, C., Löwe, B., … Wingenfeld, K. (2012). Associations of childhood trauma with hypothalamic-pituitary-adrenal function in borderline personality disorder and major depression. Psychoneuroendocrinology, 37, 1659-68.

Castellini, G., Lelli, L., Lo Sauro, C., Vignozzi, L., Maggi, M., Faravelli, C., & Ricca, V. (2012). Childhood abuse, sexual function and cortisol levels in eating disorders. Psychotherapy and Psychosomatics, 81, 380-382.

Chen, L.P., Murad, M.H., Paras, M.L., Colbenson, K.M., Sattler, A.L., Goranson, E.N., … Zirakzadeh, A. (2010). Sexual abuse and lifetime diagnosis of psychiatric disorders: systematic review and meta-analysis. Mayo Clinic Proceedings, 85, 618-629.

Chen, X., Li, S., & Kirouac, G.J. (2014). Blocking of corticotrophin releasing factor receptor-1 during footshock attenuates context fear but not theupregulation of prepro-orexin mRNA in rats. Pharmacology Biochemistry and Behavior, 31, 120,1-6.

Clarke, A.S., Ebert, M.H., Schmidt, D.E., McKinney, W.T., & Kraemer, G.W. (1999). Biogenic amine activity in response to fluoxetine and desipramine in differentially reared rhesus monkeys. Biological Psychiatry, 46, 221-228.

Clow, A., Hucklebridge, F., Stalder, T., Evans, P., & Thorn, L. (2010). The cortisol awakening response: more than a measure of HPA axis function. Neuroscience & Biobehavioral Reviews, 35, 97-103.

Cohen, P. (2008). Child development and personality disorder. Psychiatric Clinics of North America, 31, 477- 493.

Cohen, P., Brown, J., & Smaile E. (2001). Child abuse and neglect and the development of mental disorders in the general population. Developmental Psychopathology, 13, 981-999.

Collins,P.Y., Patel, V., Joestl, S.S., March, D., Insel, T.R., Daar, A.S., … Walport, M. (2011). Grand challenges in global mental health. Nature, 475, 27–30.

Connor, T.J., Brewer, C., Kelly, J.P., & Harkin, A. (2005). Acute stress suppresses pro-inflammatory cytokines TNF-alpha and IL-1 beta independent of a catecholamine-driven increase in IL-10 production. Journal of Neuroimmunology, 159, 119-128.

Corbett, B.A., Mendoza, S.P., Baym, C.L., Bunge, S.A., & Levine, S. (2008). Examining cortisol rhythmicity and responsivity to stress in children with Tourette syndrome. Psychoneuroendocrinology, 33, 810-820.

Coutinho, A.E., & Chapman, K.E. (2011). The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Molecular and Cellular Endocrinology, 335, 2-13.

Crum, A.J., Salovey, P., & Achor, S. (2013). Rethinking stress: the role of mindsets in determining the stress response. Journal of Personality and Social Psychology, 104, 716-733.

Cunningham, S.A., Kramer, M.R., & Venkat Narayan, K.M. (2014). Incidence of Childhood Obesity in the United States. New England Journal of Medicine, 370, 403-411.

Cutuli, J.J., Wiik, K.L., Herbers, J.E., Gunnar, M.R., & Masten, A.S. (2010). Cortisol function among early school-aged homeless children. Psychoneuroendocrinology, 35, 833-845.

D'Andrea, W., Ford, J., Stolbach, B., Spinazzola, J., & van der Kolk, B.A. (2012). Understanding interpersonal trauma in children: Why we need a developmentally appropriate trauma diagnosis. American Journal of Orthopsychiatry, 82, 187-200.

Dagyte, G., Van der Zee, E.A., Postema, F., Luiten, P.G., Den Boer, J.A, Trentani, A., & Meerlo P. (2009). Chronic but not acute foot-shock stress leads to temporary suppression of cell proliferation in rat hippocampus. Neuroscience, 162, 904-913.

Danese, A., & McEwen, B.S. (2012). Adverse childhood experiences, allostasis, allostatic load, and age-related disease. Physiology & Behavior, 12, 106, 29-39.

Danese, A., Pariante, C.M., Caspi, A., Taylor, A., & Poulton, R. (2007). Childhood maltreatment predicts adult inflammation in a life-course study. Proceedings of the National Academy of Sciences of the United States of America, 104, 1319-1324.

Dantzer, R., & Kelley, K.W. (2007). Twenty years of research on cytokine-induced sickness behavior. Brain, Behavior, and Immunity, 2,153-160.

Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., & Kelley, K.W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. Nature Reviews Neuroscience, 9, 46-56.

Daskalakis, N.P., Lehrner, A., & Yehuda, R. (2013a). Endocrine aspects of post-traumatic stress disorder and implications for diagnosis and treatment. Endocrinology and Metabolism Clinics of North America, 42, 503-513.

Daskalakis, N.P., Oitzl, M.S., Schächinger, H., Champagne, D.L., & de Kloet, E.R. (2012). Testing the cumulative stress and mismatch hypotheses of psychopathology in a rat model of early-life adversity. Physiology and Behavior, 106, 707-721.

Daskalakis, N.P., Yehuda, R., & Diamond, D.M. (2013b). Animal models in translational studies of PTSD. Psychoneuroendocrinology, 38, 1895-1911.

Dawes, A., Borel-Saladin, J., & Parker, Z. (2007). Measurement and monitoring. In Richter, L., Dawes, A., & Higson-Smith, C. (Eds), *Sexual abuse of young children in southern Africa.* (pp176- 205). Cape Town: HSRC Press.

De Bellis, M.D., Baum, A.S., Birmaher, B., Keshavan, M.S., Eccard, C.H., Boring, A.M., … Ryan, N.D. (1999). A.E. Bennett Research Award. Developmental traumatology. Part I: Biological stress systems. Biological Psychiatry, 45, 1259-1270.

De Bellis, M.D., Spratt, E.G., & Hooper, S.R. (2011). Neurodevelopmental biology associated with childhood sexual abuse. Journal of Child Sexual Abuse, 20, 548-587.

de Boer, S.F., & van der Gugten, J. (1987). Daily variations in plasma noradrenaline, adrenaline and corticosterone concentrations in rats. Physiology & Behavior, 40, 323–328.

Delahanty, D.L., & Nugent, N.R. (2006). Predicting PTSD prospectively based on prior trauma history and immediate biological responses. Annals of New York Academy of Sciences, 1071, 27–40.

Delahanty, D.L., Nugent, N.R., Christopher, N.C., & Walsh, M. (2000). Initial urinary epinephrine and cortisol levels predict acute PTSD symptoms in child trauma victims. Psychoneuroendocrinology, 30, 121-128.

Delanhanty, D.L., Nugent, N.R., Christopher, N.C., & Walsh, M. (2004). Initial urinary epinephrine and cortisol levels predict acute PTSD symptoms in child trauma victims. Psychoneuroendocrinology, 30, 121-128.

Desbonnet, L., Garrett, L., Clarke, G., Kiely, B., Cryan, J.F., & Dinan, T.G. (2010). Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. Neuroscience, 170, 1179-1188.

Després, J.P. (2006). Is visceral obesity the cause of the metabolic syndrome? Annals of Medicine, 38, 52-63.

Dewitte, M., De Houwer, J., Goubert, L., & Buysse, A. (2010). A multi-modal approach to the study of attachment-related distress. Biological Psychology, 85, 149-162.

Dhabhar, F.S. (2009). Enhancing versus suppressive effects of stress on immune function: Implications for immunoprotection and immunopathology. NeuroImmunoModulation, 16, 300-317.

Dhabhar, F.S., & McEwen, B.S. (2007). Bi-directional effects of stress on immune function: Possible explanations for salubrious as well as harmful effects. In R. Ader (Ed.), Psychoneurimmunology, Vol II. 4th Ed. (pp.723 – 760). San Diego: Academic Press.

Dhabhar, F.S., Malarkey, W.B., Neri, E., & McEwen, B.S. (2012). Stress-induced redistribution of immune cells--from barracks to boulevards to battlefields: a tale of three hormones--Curt Richter Award winner. Psychoneuroendocrinology, 37, 1345-1368.

Drummond, G.B., & Vowler, S.L. (2011). Statistical perspectives. Show the data, don't conceal them. Journal of Physiology, 589, 1861-1863.

Drummond, G.B., & Vowler, S.L. (2012). Statistical perspectives. Do as you would be done by: write as you would wish to read. Journal of Physiology, 590, 6251-6254.

Dunkley, A.J., Charles, K., Gray, L.J., Camosso-Stefinovic, J., Davies, M.J., & Khunti, K. (2012). Effectiveness of interventions for reducing diabetes and cardiovascular disease risk in people with metabolic syndrome: systematic

review and mixed treatment comparison meta-analysis. Diabetes, Obesity and Metabolism, 14, 616-25.

Ehlert, U. (2013). Enduring psychobiological effects of childhood adversity. Psychoneuroendocrinology, 38, 1850-1857.

Eiland L, Ramroop J, Hill MN, Manley J, McEwen BS. (2012). Chronic juvenile stress produces corticolimbic dendritic architectural remodeling and modulates emotional behavior in male and female rats. Psychoneuroendocrinology, 37, 39-47.

Eiland, L. & Romeo, R.D. (2013). Stress and the developing adolescent brain. Neuroscience, 249, 162–171.

El-Gabalawy, R., Katz, L.Y., & Sareen, J. (2010). Comorbidity and associated severity of borderline personality disorder and physical health conditions in a nationally representative sample. Psychosomatic Medicine, 72, 641-647.

Ellenbroek, B.A., & Cools, A.R. (2002). Early maternal deprivation and prepulse inhibition: the role of the postdeprivation environment. Pharmacology Biochemistry and Behavior, 73, 177-184.

Erikson, E. H. (1974). Identity: Youth and Crisis. London: Faber and Faber.

Erikson, E. H. (1977). Childhood and Society. Frogmore: Triad/Paladin.

Etter, D.W., Gauthier, J.R., McDade-Montez, E., Cloitre, M., & Carlson, E.B. (2013). Positive affect, childhood adversity, and psychopathology in psychiatric inpatients. European Journal of Psychotraumatology, 4: 20771.

Evans, G.W., Fuller-Rowell, T.E., & Doan, S.N. (2012). Childhood cumulative risk and obesity: the mediating role of self-regulatory ability. Pediatrics, 129, e68-73.

Evans, G.W., Li, D., & Whipple, S.S. (2013). Cumulative risk and child development. Psychological Bulletin, 139, 1342-1396.

Fagundes, C.P., Glaser, R., & Kiecolt-Glaser, J.K. (2013). Stressful early life experiences and immune dysregulation across the lifespan. Brain, Behavior, and Immunity, 27, 8-12.

Faturi, C.B., Tiba, P.A., Kawakami, S.E., Catallani, B., Kerstens, M., & Suchecki, D. (2010). Disruptions of the mother-infant relationship and stress-related behaviours: altered corticosterone secretion does not explain everything. Neuroscience & Biobehavioral Reviews, 34, 821-834.

Finkelhor, D. (1988). The trauma of child sexual abuse. Two models. Journal of Interpersonal Violence, 2, 348 - 366.

Finkelhor, D., Shattuck, A., Turner, H.A.,Ormrod, R., & Hamby, S.L. (2011). Polyvictimization in Developmental Context. Journal of Child & Adolescent Trauma, 4, 291-300.

Finkelhor, D., Turner, H.A., Shattuck, A., & Hamby, S.L. (2013). Violence, crime, and abuse exposure in a national sample of children and youth. An update. JAMA Pediatrics, 167, 614-621.

First, M.B., & Zimmerman, M. (2006). Including laboratory tests in DSM-V diagnostic criteria. The American Journal of Psychiatry, 163, 2041-2042.

Fischer, S., Stojek, M., & Hartzell, E. (2010). Effects of multiple forms of childhood abuse and adult sexual assault on current eating disorder symptoms. Eating Behaviors, 11, 190-192.

Fonagy, P., & Luyten, P. (2009). A developmental, mentalization-based approach to the understanding and treatment of borderline personality disorder. Developmental Psychopathology, 21, 1355-1381.

Ford, J.D., Grasso, D., Greene, C., Levine, J., Spinazzola, J., & van der Kolk B. (2013). Clinical significance of a proposed developmental trauma disorder diagnosis: results of an international survey of clinicians. Journal of Clinical Psychiatry, 74, 841-889.

Francis, L.A., Granger, D.A., & Susman, E.J. (2013). Adrenocortical regulation, eating in the absence of hunger and BMI in young children. Appetite, 64, 32-38.

Frankenburg, F.R., & Zanarini, M.C. (2006). Obesity and obesity-related illnesses in borderline patients. Journal of Personality Disorders, 20, 71-80.

Freud, S. (1987). My views on the part played by sexuality in the aetiology of the neuroses. In A. Richards (Ed.), *On psychopathology: inhibitions, symptoms and anxiety and other works. Volume 10* (pp 67-81). London: Penguin Books Ltd. (Original work published 1906).

Fries, E., Hesse. J., Hellhammer, J., & Hellhammer, D. H. (2005). A new view of hypocortisolism. Psychoneuroendocrinology, 30, 1010-1016.

Fulford, A.J., & Harbuz, M.S. (2005). An introduction to the HPA Axis. In T. Steckler., N.H. Kalin., & J.M.H.M. Reul.(Eds.), *Handbook of stress and the brain. Vol 15* (pp 43- 65). Amsterdam: Elsevier.

Fuller-Thomson, E., Bejan, R., Hunter, J.T., Grundland, T., & Brennenstuhl, S. (2012). The link between childhood sexual abuse and myocardial infarction in a population-based study. Child Abuse and Neglect, 36, 656-665.

Galic, S., Oakhill, J.S., & Steinberg, G.R. (2010). Adipose tissue as an endocrine organ. Molecular and Cellular Endocrinology, 316, 129-139.

Gareau, M.G., Jury, J., Yang, P.C., MacQueen, G., & Perdue, M.H. (2006). Neonatal maternal separation causes colonic dysfunction in rat pups including impaired host resistance. Pediatric Research, 59, 83–88.

Gee, D.G., Humphreys, K.L., Flannery, J., Goff, B., Telzer, E.H., Shapiro, M., … Tottenham, N. (2013). A developmental shift from positive to negative connectivity in human amygdala-prefrontal circuitry. The Journal of Neuroscience, 33, 4584-4593.

Gerbaix, M., Metz, L., Ringot, E., & Courteix, D. (2010). Visceral fat mass determination in rodent: validation of dual-energy x-ray absorptiometry and anthropometric techniques in fat and lean rats. Lipids in Health and Disease, 9, 140-148.

Gill, J., Vythilingam, M., & Page, G.C. (2008). Low cortisol, high DHEA, and high levels of stimulated TNF-α, and IL-6 in women with PTSD. Journal of Traumatic Stress, 21, 530-539.

Gillman, M.W., & Ludwig, D.S. (2013). How early should obesity prevention start? New England Journal of Medicine, 369, 2173-2175.

Glaser, R., & Kiecolt-Glaser, J.K. (2005). Stress-induced immune dysfunction: implications for health. Nature Reviews. Immunology, 5, 243-251.

Goldman-Mellor, S., Hamer, M., & Steptoe, A. (2012). Early-life stress and recurrent psychological distress over the lifecourse predict divergent cortisol reactivity patterns in adulthood. Psychoneuroendocrinology, 37, 1755-1768.

Goodman, M., Mascitelli, K., & Triebwasser, J. (2013). The Neurobiological Basis of Adolescent-onset Borderline Personality Disorder. Journal of Canadian Academy of Child & Adolescent Psychiatry, 22, 212-219.

Goodman, M., New, A., & Siever, L. (2004). Trauma, genes, and the neurobiology of personality disorders. Annals of the New York Academy of Sciences, 1032, 104-116.

Goodyer, I.M., Herbert, J., Tamplin, A., & Altham, P.M. (2000). First-episode major depression in adolescents. Affective, cognitive and endocrine characteristics of risk status and predictors of onset. British Journal of Psychiatry, 176, 142-149.

Gortmaker, S.L., & Taveras, E.M. (2014). Who Becomes Obese during Childhood — Clues to Prevention. New England Journal of Medicine, 370, 475-476.

Gouin, J.P., Glaser, R., Malarkey, W.B., Beversdorf, D., & Kiecolt-Glaser, J.K. (2012). Childhood abuse and inflammatory responses to daily stressors. Annals of Behavioral Medicine, 44, 287-92.

Gratz, K.L., Tull, M.T., Baruch, D.E., Bornovalova, M.A., & Lejuez, C.W. (2008). Factors associated with co-occurring borderline personality disorder among inner-city substance users: the roles of childhood maltreatment, negative affect intensity/reactivity, and emotion dysregulation. Comprehensive Psychiatry, 49, 603-615.

Greer, S. (2003). Healing the mind/body split: bringing the patient back into oncology. Integrative Cancer Therapies, 2, 5-12.

Greggersen, W., Rudolf, S., Brandt, P.W., Schulz, E., Fassbinder, E., Willenborg, B., … Schweiger, U. (2011). Intima-media thickness in women with borderline personality disorder. Psychosomatic Medicine, 73, 627-632.

Gunderson, J.G., & Lyons-Ruth, K. (2011). BPD's interpersonal hypersensitivity phenotype: a gene-environment-developmental model. Journal of Personality Disorders, 22, 22-41.

Gunnar, M.R., & Donzella, B. (2002). Social regulation of the cortisol levels in early human development. Psychoneuroendocrinology, 27, 199-220.

Gustafsson, P.E., Janlert, U., Theorell, T., & Hammarström, A. (2010). Life-course socioeconomic trajectories and diurnal cortisol regulation in adulthood. Psychoneuroendocrinology, 35, 613-623.

Hamblin, A.S. (1993). Cytokines and cytokine receptors. Oxford: Oxford University Press.

Hanahan, D., & Weinberg, R.A. (2000). The hallmarks of cancer. Cell, 100, 57–70.

Hanahan, D., & Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell, 144, 646–674.

Hancock, S.D., Menard, J.L., & Olmstead, M.C. (2005). Variations in maternal care influence vulnerability to stress-induced binge eating in female rats. Physiology and Behavior, 85, 430-439.

Hankin, B.L., Badanes, L.S., Abela, J.R., & Watamura, S.E. (2010). Hypothalamic-pituitary-adrenal axis dysregulation in dysphoric children and adolescents: cortisol reactivity to psychosocial stress from preschool through middle adolescence. *Biological Psychiatry*, *68,* 484-490.

Harper, J.M. & Austad, S.N. (2000). Fecal glucocorticoids: A noninvasive method of measuring adrenal activity in wild and captive rodents. Physiological and Biochemical Zoology, 73,12-22.

Harrison, N.A., Brydon, L., Walker, C., Gray, M.A., Steptoe, A., & Critchley, H.D. (2009). Inflammation causes mood changes through alterations in subgenual cingulated activity and mesolimbic connectivity. Biological Psychiatry, 66, 407-14.

Harwood, H.J. Jr. (2012). The adipocyte as an endocrine organ in the regulation of metabolic homeostasis. Neuropharmacology, 63, 57-75.

Hastings, P.D., Shirtcliff, E.A., Klimes-Dougan, B., Allison, A.L., Derose, L., Kendziora, K.T., … Zahn-Waxler, C. (2011). Allostasis and the development of internalizing and externalizing problems: changing relations with physiological systems across adolescence. Developmental Psychopathology, 23, 1149-1165.

Hayashi-Takagi, A., Vawter, M.P., & Iwamoto, K. (2013). Peripheral biomarkers revisited: Integrative profiling of peripheral samples for psychiatric research. Biological Psychiatry, doi:http://dx.doi.org/10.1016/j.biopsych.2013.09.035

Hayley, S., Poulter, M.O., Merali, Z., & Anisman, H. (2005). The pathogenesis of clinical depression: stressor- and cytokine-induced alterations of neuroplasticity. Neuroscience, 135, 659-678.

Heffernan, K., & Cloitre, M. (2000). A comparison of posttraumatic stress disorder with and without borderline personality disorder among women a history of childhood sexual abuse: etiological and clinical characteristics. The Journal of Nervous and Mental Disease, 189, 589-595.

Heim, C., Ehlert, U., & Hellhammer, D.H. (2000). The potential role of hypocortisolism in the pathophysiology of stress-related bodily disorders. Psychoneuroendocrinology, *25*, 1- 35.

Heim, C., Newport, D.J., Mletzko, T., Miller, A.H., & Nemeroff, C.B. (2008). The link between childhood trauma and depression: Insights from HPA axis studies in humans. Psychoneuroendocrinology, 33, 693-710.

Heim, C., Plotsky, P.M., & Nemeroff, C.B. (2004). Importance of studying the contributions of early adverse experience to neurobiological findings in depression. Neuropsychopharmacology, 29, 641-648.

Heim, C., Shugart, M., Craighead, W.E., & Nemeroff, C.B. (2010). Neurobiological and psychiatric consequences of child abuse and neglect. Developmental Psychobiology, 52, 671-690.

Helmreich, D.L., Parfitt, D.B., Lu, X.Y., Akil, H., & Watson, S.J. (2005). Relation between the hypothalamic-pituitary-thyroid (HPT) axis and the hypothalamic-pituitary-adrenal (HPA) axis during repeated stress. Neuroendocrinology, 81, 183-192.

Hendricks, J. H., Black, D., & Kaplan, T. (1993). When Father Kills Mother. London: Routledge.

Henningsen, K., Dyrvig, M., Bouzinova, E.V., Christiansen, S., Christensen, T., Andreasen, J.T., …, Wiborg, O. (2012). Low maternal care exacerbates adult stress susceptibility in the chronic mild stress rat model of depression. Behavioral Pharmacology, 23, 735-743.

Herman, J. (2012). COMMENT. CPTSD is a Distinct Entity: Comment on Resick et al. Journal of Traumatic Stress. 25, 256–257.

Herman, J.L., Perry, J.C., & van der Kolk, B.A. (1989). Childhood trauma in borderline personality disorder. American Journal of Psychiatry, 146, 490-495.

Herman, J.P., & Spencer, R. (1998). Regulation of Hippocampal Glucocorticoid Receptor Gene Transcription and Protein Expression in Vivo. The Journal of Neuroscience, 18, 7462–7473.

Herman, J.P., Figueiredo, H., Mueller, N.K., Ulrich-Lai, Y., Ostrander, M.M., Choi, D.C., & Cullinan, W.E. (2003). Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo–pituitary–adrenocortical responsiveness. Frontiers in Neuroendocrinology, 24, 151–180.

Hermes, G.L., Rosenthal, L., Montag, A., & McClintock, M.K. (2005). Social isolation and the inflammatory response: sex differences in the enduring effects of a prior stressor. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology, 290, R273-R282.

Holscher, C. (1999). Stress impairs performance in spatial water maze learning tasks. Behavioural Brain Research, 100, 225-235.

Hostinar, C.E., & Gunnar, M.R. (2013). Future directions in the study of social relationships as regulators of the HPA Axis across development. Journal of Clinical Child & Adolescent Psychology, 42, 564-575.

Hostinar, C.E., Sullivan, R.M., & Gunnar, M.R. (2013). Psychobiological Mechanisms Underlying the Social Buffering of the Hypothalamic-Pituitary-Adrenocortical

Axis: A Review of Animal Models and Human Studies across development. Psychological Bulletin, 140, 256-282.

Hueston, C.M., & Deak, T. (2013). The inflamed axis: The interaction between stress, hormones, and the expression of inflammatory-related genes within key structures comprising the hypothalamic-pituitary-adrenal axis. Physiology & Behavior, 124, 77-91

Hueston, C.M., Barnum, C.J., Eberle, J.A., Ferraioli, F.J., Buck, H.M., & Deak, T. (2011). Stress-dependent changes in neuroinflammatory markers observed after common laboratory stressors are not seen following acute social defeat of the Sprague Dawley rat. Physiology & Behavior, 104, 187-198.

Hyland, M.E., Alkhalaf, A.M., & Whalley, B. (2012). Beating and insulting children as a risk for adult cancer, cardiac disease and asthma. Journal of behavioral Medicine, 36, 632-640.

Hyman, S.E. (2009). How adversity gets under the skin. Nature Neuroscience, 12, 241-243.

Ibrahim, M.M. (2009). Subcutaneous and visceral adipose tissue: structural and functional differences. Obesity Reviews, 11, 11-8.

Insel, T.R. (2007). The arrival of preemptive psychiatry. Early Intervention in Psychiatry, 1, 5-6.

Irwin, M.R. (2008). Human psychoneuroimmunology: 20 years of discovery. Brain, Behavior, and Immunity, 22, 129-139.

Irwin, M.R., & Miller, A.H. (2007). Depressive disorders and immunity: 20 years of progress and discovery. Brain, Behavior, and Immunity, 21, 374-383.

Izawa, S., Miki, K., Liu, X., & Ogawa, N. (2013a). The diurnal patterns of salivary interleukin-6 and C-reactive protein in healthy young adults. Brain, Behavior, and Immunity, 27, 38-41.

Izawa, S., Sugaya, N., Kimura, K., Ogawa, N., Yamada, K.C., Shirotsuki, K., …Nomura, S. (2013b). An increase in salivary interleukin-6 level following acute psychosocial stress and its biological correlates in healthy young adults. Biological Psychology, 94, 249–254.

Jahng, W.J. (2011). An animal model of eating disorders associated with stressful experience in early life. Hormones and Behavior, 59, 213-220.

Johnson, J.D., O'Conner, K.A., Hansen, M.K., Watkins, L.R., & Maier, S.F. (2003). Effects of prior stress on LPS-induced cytokine and sickness responses. American Journal of Physiology – Regulatory, Integrative and Comparative Physiology, 284, R422-R432.

Johnson, J.D., O'Connor, K.A., Deak, T., Spencer, R.L., Watkins, L.R., & Maier, S.F. (2002). Prior stressor exposure primes the HPA axis. Psychoneuroendocrinology, 27, 353-365.

Johnson, J.G., Smailes, E.M., Cohen, P., Brown, J., & Bernstein, D.P. (2000). Associations between four types of childhood neglect and personality disorder symptoms during adolescence and early adulthood: findings of a community-based longitudinal study. Journal of Personality Disorders, 14, 171-187.

Jonkman, C.S., Verlinden, E., Bolle, E.A., Boer, F., & Lindauer, R.J. (2013). Traumatic stress symptomatology after child maltreatment and single traumatic events: different profiles. Journal of Traumatic Stress, 26, 225-232.

Jovanovic, T., Blanding, N.Q., Norrholm, S.D., Duncan, E., Bradley, B., & Ressler, K.J. (2009). Childhood abuse is associated with increased startle reactivity in adulthood. Depression and Anxiety, 26, 1018-1026.

Juruena, M.F. (2013). Early-life stress and HPA axis trigger recurrent adulthood depression. Epilepsy & Behavior, pii: S1525-5050(13)00553-2.

Juster, R., Sindi, S., Marin, M., Perna, A., Hashemi, A., Pruessner, J.C., & Lupien, S.J. (2011). A clinical allostatic load index is associated with burnout symptoms and hypocortisolemic profiles in healthy workers. Psychoneuroendocrinology, 36, 797-805.

Kahl, K.G., Bens, S., Ziegler, K., Rudolf, A., Dibbelt, L., Kordon, A., & Schweiger, U. (2006). Cortisol, the Cortisol-Dehydroepiandosterone Ratio, and Pro-Inflammatory Cytokines in patients with current Major Depressive Disorder comorbid with Borderline Personality Disorder. Biological Psychiatry, 59; 667-671.

Kahl, K.G., Bester, M., Greggersen, W., Rudolf, S., Dibbelt, L., Stoeckelhuber, B.M., … Schweiger, U. (2005). Visceral fat deposition and insulin sensitivity in depressed women with and without comorbid borderline personality disorder. Psychosomatic Medicine, 67, 407-412.

Kalsbeek, A., van der Spek, R., Lei, J., Endert, E., Buijs, R.M., & Fliers, E. (2012). Circadian rhythms in the hypothalamo-pituitary-adrenal (HPA) axis. Molecular and Cellular Endocrinology, 349, 20-29.

Kaplan, H.I., & Sadock, B.J. (1988). Synopsis of Psychiatry. 5th ed, Williams and Wilkins. Baltimore.

Kapur, S., Phillips, A.G., & Insel, T.R. (2012). Why has it taken so long for biological psychiatry to develop clinical tests and what to do about it? Molecular Psychiatry, 17, 1174–1179.

Karlamangla, A.S., Friedman, E.M., Seeman, T.E., Stawksi, R.S., & Almeida, D.M. (2013). Daytime trajectories of cortisol: demographic and socioeconomic differences--findings from the National Study of Daily Experiences. Psychoneuroendocrinology, 38, 2585-2597.

Katz, D.A., Sprang, G., & Cooke, C. (2012). The Cost of Chronic Stress in Childhood: Understanding and Applying the Concept of Allostatic Load. Psychodynamic Psychiatry, 40, 469-480.

Kaufman, J., Birmaher, B., Perel, J., Dahl, R.E., Moreci, P., Nelson, B., … Ryan, N.D. (1997). The corticotropin-releasing hormone challenge in depressed abused, depressed nonabused, and normal control children. *Biological Psychiatry*, *42*, 669-679.

Kavanaugh, B., Holler, K., & Selke, G. (2013). A Neuropsychological Profile of Childhood Maltreatment within an Adolescent Inpatient Sample. Applied Neuropsychology: Child, DOI:10.1080/21622965.2013.789964

Keeshin, B.R., Strawn, J.R., Out, D., Granger, D.A., & Putnam, F.W. (2013). Cortisol awakening response in adolescents with acute sexual abuse related posttraumatic stress disorder. Depression and Anxiety, 31, 107-114.

Kerkhof, G.F., Leunissen, R.W.J., & Hokken-Koelega, A.C.S. (2012). Early Origins of the Metabolic Syndrome: Role of Small Size at Birth, Early Postnatal Weight

Gain, and Adult IGF-I. The Journal of Clinical Endocrinology & Metabolism, 97, 2637-2643.

Kernberg, O.F. (1993). The Psychotherapeutic Treatment of Borderline Patients. In J. Paris (Ed.), Borderline Personality Disorder. Etiology and Treatment (pp. 261 - 284). Washington, DC: American Psychiatric Press Inc

Kessler, R.C., Aguilar-Gaxiola, S., Alonso, J., Chatterji, S., Lee, S., Ormel, J., … Wang, P.S. (2009). The global burden of mental disorders: an update from the WHO World Mental Health (WMH) surveys. Epidemiologia e Psichiatria Sociale, 18, 23-33.

Khanfer, R., Phillips, A.C., Carroll, D., & Lord, J.M. (2010). Altered Human Neutrophil Function in Response to Acute Psychological Stress. Psychosomatic Medicine, 7, 636-640.

Kinderman, P., Schwannauer, M., Pontin, E., & Tai, S. (2013). Psychological Processes Mediate the Impact of Familial Risk, Social Circumstances and Life Events on Mental Health. PLoS ONE 8, e76564.

Klein, M. (1955). On Identification. In Envy and Gratitude and Other Works 1946 - 1963 (pp. 141 - 175). London: Virago Press Limited (1988).

Korosi, A., Naninck, E.F., Oomen, C.A., Schouten, M., Krugers, H., Fitzsimons, C., & Lucassen, P.J. (2012). Early-life stress mediated modulation of adult neurogenesis and behavior. Behavioral Brain Research, 227, 400-409.

Korte, S.M., Koolhaas, J.M., Wingfield, J.C., & McEwen, B.S. (2005). The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. Neuroscience and Biobehavioral reviews, 29, 3-38.

Kudielka, B.M., Schommer, N.C., Hellhammer, D.H., & Kirschbaum, C. (2004). Acute HPA axis responses, heart rate, and mood changes to psychosocial stress (TSST) in humans at different times of day. Psychoneuroendocrinology, 29, 983-992.

Kyrou, I., & Tsigos, C. (2009). Stress hormones: physiological stress and regulation of metabolism. Current Opinion in Pharmacology, 9, 787-793.

Labudda, K., Kreisel, S., Beblo, T., Mertens, M., Kurlandchikov, O., Bien, C.G., Driessen, M., & Woermann, F.G. (2013). Mesiotemporal Volume Loss Associated with Disorder Severity: A VBM Study in Borderline Personality Disorder. PLoS One, 8, e83677.

Ladd, C.O., Huot, R.L., Thrivikraman, K.V., Nemeroff, C.B., & Plotsky, P.M. (2004). Long-term adaptations in glucorticoid receptor amd mineralocorticoid receptor mRNA and negative feedback on the hypothalamo-pituitary-adrenal axis following neonatal maternal separation. Biological Psychiatry, 55, 367-375.

Lamers, F., Vogelzangs, N., Merikangas, K.R., de Jonge, P., Beekman, A.T., & Penninx, B.W. (2013). Evidence for a differential role of HPA-axis function, inflammation and metabolic syndrome in melancholic versus atypical depression. Molecular Psychiatry, 18, 692-699.

Lang, U.E., & Borgwardt, S. (2013). Molecular mechanisms of depression: perspectives on new treatment strategies. Cellular Physiology and Biochemistry, 31, 761-777.

Lange, C., Zschucke, E., Ising, M., Uhr, M., Bermpohl, F., & Adli, M. (2013). Evidence for a normal HPA axis response to psychosocial stress in patients remitted from depression. Psychoneuroendocrinology, 38, 2729-2736.

Lansford, J.E., Dodge, K.A., Pettit, G.S., & Bates, J.E. (2010). Does physical abuse in early childhood predict substance use in adolescence and early adulthood? Child Maltreatment, 15,190-194.

Lee, R.J., Hempel, J., TenHarmsel, A., Liu, T., Mathe, A.A., & Klock, A. (2012). The neuroendocrinology of childhood trauma in personality disorder. Psychoneuroendocrinology, 37, 78-86.

Leenarts, L.E., Diehle, J., Doreleijers, T.A., Jansma, E.P., & Lindauer, R.J. (2013). Evidence-based treatments for children with trauma-related psychopathology as a result of childhood maltreatment: a systematic review. European Child and Adolescent Psychiatry, 22, 269-283.

Lehmann, J., Russig, H., Joram, F., & Pryce, C.R. (2002). Effect of a single maternal separation at different pup ages on the corticosterone stress response in adult and aged pups. Pharmacology Biochemistry and Behavior, 73, 141-145.

Levine, A.B., Levine, L.M., & Levine, T.B. (2013). Posttraumatic stress disorder and cardiometabolic disease. Cardiology, 127, 1-19.

Levine, S. (1957). Infantile experience and resistance to physiological stress. Science, 30, 405.

Levine, S. (2000). Influence of psychological variables on the activity of the hypothalamic-pituitary-adrenal axis. European Journal of Pharmacology, 405, 149-160.

Levine, S. (2005). Developmental determinants of sensitivity and resistance to stress. Psychoneuroendocrinology, 30, 939-946

Levine, S. Huchton, D.M., Wiener, S.G., Rosenfeld, P., (1992). Time course of the

effect of maternal deprivation on the hypothalamic-pituitary-adrenal axis in the infant rat. Developmental Psychobiology, 24, 547-558

Levine, S., & Lewis, G.W. (1959). Critical Period for Effects of Infantile experience on maturation of stress response. Science, 129, 42-43.

Levine, S., Huchton, D.M., Wiener, S.G., & Rosenfeld, P. (1992). Time course of the effect of maternal deprivation on the hypothalamic-pituitary-adrenal axis in the infant rat. Developmental Psychobiology, 24, 547-558.

Linares, L.O., Stovall-McClough, K.C., Li, M., Morin, N., Silva, R., Albert, A., & Cloitre, M. (2008). Salivary cortisol in foster children: a pilot study. Child Abuse and Neglect. 32, 665-670.

Litvin, Y., Tovote, P., Pentkowski, N.S., Zeyda, T., King, L.B., Vasconcellos, A.J., … & Blanchard, R.J. (2010). Maternal separation modulates short –term behavioral and physiological indices of the stress response. Hormones and Behavior, 58, 241-249.

Logan, J.G., & Barksdale, D.J. (2008). Allostasis and allostatic load: expanding the discourse on stress and cardiovascular disease. Journal of Clinical Nursing, 17, 201-208.

Loman, M.M., & Gunnar, M.R (2010). Early experience and the development of stress reactivity and regulation in children. Neuroscience and Biobehavioral Reviews, 34, 867-876.

Lorenzetti, V., Allen, N.B., Fornito, A., & Yücel, M. (2009). Structural brain abnormalities in major depressive disorder: a selective review of recent MRI studies. Journal of Affective Disorders, 117, 1–17.

Louvart, H., Maccari, S., Ducrocq, F., Thomas, P. & Darnaudery, M. (2005). Long-term behavioural alterations in female rats after a single intense footshock followed by situational reminders. Psychoneuroendocrinology, 30, 316-324.

Lovallo, W.R., Farag, N.H., Sorocco, K.H., Cohoon, A.J., & Vincent, A.S. (2012). Lifetime adversity leads to blunted stress axis reactivity: studies from the Oklahoma Family Health Patterns Project. Biological Psychiatry, 71, 344-349.

Lu, X.T., Zhao, Y.X., Zhang, Y., & Jiang, F. (2013). Psychological stress, vascular inflammation, and atherogenesis: potential roles of circulating cytokines. Journal of Cardiovascular Pharmacology, 62, 6-12.

Lupien, S.J., King, S., Meaney, M.J., & McEwen, B.S. (2000). Child's stress hormone levels correlate with mother's socioeconomic status and depressive state. Biological Psychiatry, 48, 976-980.

Lynch, W.J., Mangini, L.D., & Taylor, J.R. (2005). Neonatal isolation stress potentiates cocaine seeking behaviour in adult male and female rats. Neuropsychopharmacology, 30, 322-329.

Lyons, D.M., Parker, K.J., & Schatzberg, A.F. (2010). Animal models of early life stress: Implications for understanding resilience. Developmental Psychobiology, 52, 402-410.

MacMillan, H.L., Georgiades, K., Duku, E.K., Shea, A., Steiner, M., Niec, A., … Schmidt LA. (2009). Cortisol response to stress in female youths exposed to childhood maltreatment: results of the youth mood project. Biological Psychiatry, 66, 62-68.

Macrì, S., Zoratto, F., & Laviola, G. (2011). Early-stress regulates resilience, vulnerability and experimental validity in laboratory rodents through mother-offspring hormonal transfer. Neuroscience & Biobehavioral Reviews, 35, 1534–1543.

Maes, M., Scharpé, S., Meltzer, H.Y., Bosmans, E., Suy, E., Calabrese, J., & Cosyns, P. (1993). Relationships between interleukin-6 activity, acute phase proteins, and function of the hypothalamic-pituitary-adrenal axis in severe depression. Psychiatry Research, 49, 11-27.

Mahler, M.S.; Pine, F., & Bergman, A. (1975). The Psychological Birth of the Human Infant. London: Hutchinson.

Maletic, V., Robinson, M., Oakes, T., Iyengar, S., Ball, S.G., & Russell, J. (2007). Neurobiology of depression: an integrated view of key findings. International Journal of Clinical Practice, 61, 2030-2040.

Mason, J.W., Wang, S., Yehuda, R., Lubin, H., Johnson, D., Bremner, J.D., … Southwick, S. (2002). Marked lability in urinary cortisol levels in subgroups of combat veterans with posttraumatic stress disorder during an intensive exposure treatment program. Psychosomatic Medicine, 64, 238-246.

Mathews S, Abrahams N, & Jewkes R. (2013). Exploring mental health adjustment of children post sexual assault in South Africa. Journal of Child Sexual Abuse, 22, 639-657.

Maughan, B., & Rutter, M. (1997), Retrospective reporting of childhood adversity: issues in assessing long-term recall. Journal of Personality Disorders, 11, 19-33.

McCrory, E., De Brito, S.A., & Viding, E. (2012). The link between child abuse and psychopathology: a review of neurobiological and genetic research. Journal of the Royal Society of Medicine, 105, 151-156.

McDade, T.W., Hokea, M., Borjac, J.B., Adair, L.S., & Kuzawa, C. (2013). Do environments in infancy moderate the association between stress and inflammation in adulthood? Initial evidence from a birth cohort in the Philippines. Brain, Behavior, and Immunity, 31, 23–30.

McEwen, B.S. (1998a). Protective and damaging effects of stress mediators. New England Journal of Medicine, 338, 171-179.

McEwen, B.S. (1998b). Stress, adaptation, and disease. Allostasis and allostatic load. Annals of New York Academy of Sciences, 840, 33-44.

McEwen, B.S. (2000). The neurobiology of stress: from serendipity to clinical relevance. Brain Research, 886, 172-189.

McEwen, B. S. (2003a). Early life influences on life-long patterns of behaviour and health. Mental Retardation and Developmental Disabilities Research Reviews, 9, 149-154.

McEwen, B.S. (2003b). Mood disorders and allostatic load. Biological Psychiatry, 54, 200-207.

McEwen, B. S. (2005). "Stressed or stressed out: What is the difference?" Journal of Psychiatry and Neuroscience, 30, 315-318.

McEwen, B.S. (2006). Protective and damaging effects of stress mediators: central role of the brain. Dialogues in Clinical Neuroscience, 8, 367-381.

McEwen, B.S. (2006). Protective and damaging effects of stress mediators: central role of the brain. Dialogues of Clinical Neuroscience, 8, 367-381. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3181832/pdf/DialoguesClinNeurosci-8-367.pdf

McEwen, B.S. (2007). Physiology and neurobiology of stress and adaptation: central role of the brain. Physiological Reviews, 87, 873-904.

McEwen, B.S., & Gianaros, P.J. (2010). Central role of the brain in stress and adaptation: Links to socioeconomic status, health, and disease. Annals of the New York Academy of Sciences, 1186, 190-222.

McEwen, B.S., Biron, C.A., Brunson, K.W., Bulloch, K., Chambers, W.H., Dhabhar, F.S., … Weiss, J.M. (1997). The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. Brain Research Reviews, 23, 79-133.

McGowan, P.O. (2013). Epigenomic Mechanisms of Early Adversity and HPA Dysfunction: Considerations for PTSD Research. Frontiers in Psychiatry, 4, 110.

McGowan, P.O., Sasaki, A., D'Alessio, A.C., Dymov, S., Labonté, B., Szyf, M., … Meaney, M.J. (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nature Neuroscience, 12, 342–348.

McIntyre, R.S, Soczynska, J.K., Konarski, J.Z., Woldeyohannes, H.O., Law, C.W.Y., Miranda, A., & Kennedy, S.H. (2007). Should depressive illness be reclassified as "metabolic syndrome type II"? Annals of Clinical Psychiatry, 19, 257-264.

Meaney, M.J. (2001). Maternal Care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annual Review of Neuroscience, 24, 1161-1199.

Meiring, R.M., Avidon, I., Norris, S.A., & McVeigh, J.A. (2013). A two-year history of high bone loading physical activity attenuates ethnic differences in bone strength and geometry in pre-/early pubertal children from a low-middle income country. Bone, 57, 522-530.

Miller, A.H., Maletic, V., & Raison, C.L. (2009). Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biological Psychiatry, 65, 732-741.

Miller, A.H., Pariante, C.M., & Pearce, B.D. (1999). Efffects of cytokines on glucocorticoid receptor expression and function. Glucocorticoid resistance and relevance to depression. In R Dantzer, EE Wollman & R Yirmiya (Eds.), *Cytokines. Stress, and Depression. Advances in Experimental Medicine and Biology. Vol 461* (pp. 107-116). New York: Kluwer Academic.

Miller, A.L., Clifford, C., Sturza, J., Rosenblum, K., Vazquez, D.M., Kaciroti, N., & Lumeng, J.C. (2013). Blunted cortisol response to stress is associated with higher body mass index in low-income preschool-aged children. Psychoneuroendocrinology, 38, 2611-2617.

Miller, B.J., Mellor, A., & Buckley, P. (2013). Total and differential white blood cell counts, high-sensitivity C-reactive protein, and the metabolic syndrome in non-affective psychoses. Brain, Behavior, and Immunity, 31, 82-89.

Miller, G.E., & Cole, S.W. (2012). Clustering of Depression and Inflammation in Adolescents Previously Exposed to Childhood Adversity. Biological Psychiatry, 72, 34–40.

Miller, G.E., Cohen, S., & Ritchey, A.K. (2002). Chronic psychological stress and the regulation of pro-inflammatory cytokines: a glucocorticoid-resistance model. Health Psychology, 21, 531-541.

Mills, N.T., Scott, J.G., Wray, N.R., Cohen-Woods, S., & Baune, B.T. (2013). Research review: The role of cytokines in depression in adolescents: a systematic review. Journal of Child Psychology and Psychiatry, 54, 816-835.

Moore, N.L.T., Gauchan, S., & Genovese, R.F. (2014). Adolescent traumatic stress experience results in less robust conditioned fear and post-extinction fear cue responses in adult rats. Pharmacology Biochemistry and Behavior, 120C, 17-24

Morris, M.C., & Rao, U. (2013). Psychobiology of PTSD in the acute aftermath of trauma: Integrating research on coping, HPA function and sympathetic nervous system activity. Asian Journal of Psychiatry, 6, 3-21.

Muller, D., Errington, S., Szabo, C.P., Pitts, N., & Jacklin, L., (2014). Cortisol and IL-6 responses to stress in female children presenting at a sexual abuse clinic. Journal of Child and Adolescent Trauma, in press.

Murgatroyd, C. (2014). Epigenetic programming of neuroendocrine systems during early life. Experimental Physiology, 99, 62-65.

Musselman, D.L., Miller, A.H., Porter, M.R., Manatunga, A., Gao, F., Penna, S., … Nemeroff, C.B. (2001). Higher than normal plasma interleukin-6 concentrations

in cancer patients with depression: preliminary findings. The American Journal of Psychiatry, 158, 1252-1257.

Nader, K. (2011). The Assessment of Associated Features Important to Understanding Childhood Trauma. Journal of Child & Adolescent Trauma, 4, 259-273.

Nader, K., & Weems, C.F. (2011). Understanding and Assessing Cortisol Levels in Children and Adolescents. Journal of Child & Adolescent Trauma, 4, 318-338.

Newman, J.D., & Harris, J.C. (2009). The scientific contributions of Paul D. MacLean (1913-2007). Journal of Nervous and Mental Disease, 197, 3-5.

Nijm, J., Kristenson, M., Olsson, A.G., & Jonasson, L. (2007). Impaired cortisol response to acute stressors in patients with coronary disease. Implications for inflammatory activity. Journal of Internal Medicine, 62, 375-384.

O'Brien, S.M., Scott, L.V., & Dinan, T.G. (2004). Cytokines: abnormalities in major depression and implications for pharmacological treatment. Human Psychopharmacology, 19, 397-403.

O'Conner, K.A., Johnson, J.D., Hansen, M.K., Frank, J.L.W., Maksimova, E., Watkins, L.R. & Maier, S.F. (2003). Peripheral and central proinflammatory cytokine response to a severe acute stressor. Brain Research, 991, 123-132.

O'Leary, M.M., Loney, B.R., & Eckel, L.A. (2007). Gender differences in the association between psychopathic personality traits and cortisol response to induced stress. Psychoneuroendocrinology, 32, 183-191.

O'Mahony, S.M., Marchesi, J.R., Scully, P., Codling, C., Ceolho, A., Quigley, E.M.M., … Dinan, T.G. (2009). Early life stress alters behaviour, immunity, and

microbiota in rats: Implications for irritable bowel syndrome and psychiatric illnesses. Biological Psychiatry, 65, 263-267.

Ogden, C.L., Carroll, M.D.; Kit, B.K., & Flegal, K.M. (2014). Prevalence of Childhood and Adult Obesity in the United States, 2011-2012. JAMA, 311, 806-814.

O'Malley, D., Liston, M., Hyland, NP., Dinan, T.G., & Cryan, J.F. (2011). Colonic soluble mediators from the maternal separation model of irritable bowel syndrome activate submucosal neurons via an interleukin-6-dependent mechanism. American Journal of Physiology - Gastrointestinal and Liver Physiology, 300, G241-G252.

Owens, M., Herbert, J., Jones, P.B., Sahakian, B.J., Wilkinson, P.O., Dunn, V.J., Croudace, T.J., & Goodyer, I.M. (2014). Elevated morning cortisol is a stratified population-level biomarker for major depression in boys only with high depressive symptoms. Proceedings of the National Academy of Sciences, 111, 3638-3643

Pace, T.W.W., Mletzko, T.C., Alagbe, O., Musselman, D.L., Nemeroff, C.B., Miller, A.H., & Heim, C.M. (2006). Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress. The American Journal of Psychiatry, 163, 1630-1633.

Pajer, K.1., Andrus, B.M., Gardner, W., Lourie, A., Strange, B., Campo, J., … Redei EE. (2012). Discovery of blood transcriptomic markers for depression in animal models and pilot validation in subjects with early-onset major depression. Translational Psychiatry, 2, e101.

Pally, R. (2002). The neurobiology of borderline personality disorder: the synergy of "nature and nurture". Journal of Psychiatric Practice, 8, 133-142.

Paras, M.L., Murad, M.H., Chen, L.P., Goranson, E.N., Sattler, A.L., Colbenson, K.M., … Zirakzadeh, A. (2009). Sexual abuse and lifetime diagnosis of somatic disorders: a systematic review and meta-analysis. JAMA, 302, 550-561.

Paris, J., & Zweig-Frank, H. (1993). Parental bonding in Borderline Personality Disorder. In J. Paris (Ed.), Borderline Personality Disorder. Etiology and Treatment (pp. 141 - 159). Washington, DC: American Psychiatric Press Inc

Paris. J. (Ed.). (1993). Introduction. In J. Paris (Ed.), Borderline Personality Disorder. Etiology and Treatment (pp. 3-12). Washington, DC: American Psychiatric Press Inc

Patterson, Z.R. & Abizaid, A. (2013). Stress induced obesity: lessons from rodent models of stress. Frontiers in Neuroscience, 7, 130.

Penninx, B.W., Milaneschi, Y., Lamers, F., & Vogelzangs, N. (2013). Understanding the somatic consequences of depression: biological mechanisms and the role of depression symptom profile. BMC Medicine. 11, 129.

Perroud, N., Dayer, A., Piguet, C., Nallet, A., Favre, S., Malafosse, A., & Aubry, J.M. (2014). Childhood maltreatment and methylation of the glucocorticoid receptor gene NR3C1 in bipolar disorder. British Journal of Psychiatry; 204, 30–5.

Pervanidou, P. (2008). Biology of post-traumatic stress disorder in childhood and adolescence. Journal of Neuroendocrinology, 20, 632-638.

Pervanidou, P., & Chrousos, G.P. (2012a). Metabolic consequences of stress during childhood and adolescence. Metabolism, 61, 611-619.

Pervanidou, P., & Chrousos, G.P. (2012b). Posttraumatic Stress Disorder in Children and Adolescents: Neuroendocrine Perspectives. Science Signal. 5, pt6.

Pervanidou, P., Kolaitis, G., Charitaki, S., Margeli, A., Ferentinos, S., Bakoula, C., …Chrousos, G.P. (2007). Elevated morning serum interleukin (IL)-6 or evening salivary cortisol concentrations predict posttraumatic stress disorder in children and adolescents six months after a motor vehicle accident. Psychoneuroendocrinology, 32, 991-999.

Petrowski, K., Herold, U., Joraschky, P., Wittchen, H.U., & Kirschbaum, C. (2010). A striking pattern of cortisol non-responsiveness to psychosocial stress in patients with panic disorder with concurrent normal cortisol awakening responses. Psychoneuroendocrinology, 35, 414-421.

Petterino, C., & Argentino-Storino, A. (2006). Clinical chemistry and haematology historical data in control Sprague-Dawley rats from pre-clinical toxicity studies. Experimental and Toxicologic Pathology, 57, 213–219.

Phelan, N., O'Connor, A., Kyaw Tun, T., Correia, N., Boran, G., Roche, H.M., & Gibney, J. (2013). Leucocytosis in women with polycystic ovary syndrome (PCOS) is incompletely explained by obesity and insulin resistance. Clinical Endocrinology, 78, 107-113.

Phillips, A.C., Ginty, A.T., & Hughes, B.M (2013). The other side of the coin: Blunted cardiovascular and cortisol reactivity are associated with negative health outcomes. International Journal of Psychophysiology, 90, 1-7.

Plotsky, P.M., & Meaney, M.J. (1993). Early, postnatal experience alters hypothalmiccorticotropin-releasing factor (CRF) mRNA, median eminence CRF

content and stress-induced release in adult rats. Brain Research. Molecular Brain Research, 18, 195-200.

Poole, L., Kidd, T., Leigh, E., Ronaldson, A., Jahangiri, M., & Steptoe, A. (2013). Depression, C-reactive protein and length of post-operative hospital stay in coronary artery bypass graft surgery patients. Brain, Behavior, and Immunity. pii: S0889-1591(13)00539-4.

Powers, A.D., & Oltmanns, T.F. (2012). Personality disorders and physical health: a longitudinal examination of physical functioning, healthcare utilization, and health-related behaviors in middle-aged adults. Journal of Personality Disorders, 26, 524-538.

Pryce, C.R., Ruedi-Bettschen, D., Dettling, A.C., & Feldon, J. (2002). Early life stress: Long-term physiological impact in rodents and primates. Physiology, 17, 150-155.

Pryce, C.R., Rüedi-Bettschen, D., Dettling, A.C., Weston, A., Russig, H., Ferger, B., & Feldon, J. (2005). Long-term effects of early-life environmental manipulations in rodents and primates: Potential animal models in depression research. Neuroscience & Biobehavioral Reviews, 29, 649-674.

Pugh, C.R., Fleshner, M., Watkins, L.R., Maier, S.F., & Rudy, J.W. (2001). The immune system and memory consolidation: a role for the cytokine 1L-beta. Neuroscience and Behavioural Reviews, 25, 29-41.

Pyter, L.M., Yang, L., da Rocha, J.M., & Engeland, C.G. (2014). The effects of social isolation on wound healing mechanisms in mice. Physiology & Behavior, 127, 64-70.

Rabasa, C., Muñoz-Abellán, C., Daviu, N., Nadal, R., & Armario, A. (2011). Repeated exposure to immobilization or two different footshock intensities reveals differential adaptation of the hypothalamic–pituitary–adrenal axis. Physiology & Behavior, 103, 125–133.

Räikkönen, K., Keltikangas-Järvinen, L., Adlercreutz, H., & Hautanen, A. (1996). Psychosocial stress and the insulin resistance syndrome. Metabolism, 45, 1533-1538.

Raineki, C., Morirceau, S., & Sullivan, R.M., (2010). Developing a neurobehavioral animal model of infant attachment to an abusive caregiver. Biological Psychiatry, 67, 1137-1145.

Raison, C.L., & Miller, A.H. (2003). When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. American Journal of Psychiatry, 160, 1554-1565.

Raison, C.L., & Miller, A.H. (2011). Is Depression an Inflammatory Disorder? Current Psychiatry Reports, 13, 467-475.

Raison, C.L., & Miller, A.H. (2013a). Do Cytokines Really Sing the Blues? Cerebrum, 10, Retrieved from: http://www.dana.org/news/cerebrum/detail.aspx?id=44322

Raison, C.L., & Miller, A.H. (2013b). Malaise, melancholia and madness: the evolutionary legacy of an inflammatory bias. Brain, Behavior, and Immunity, 31, 1-8.

Raison, C.L., Capuron, L., & Miller, A.H. (2006). Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends in Immunology, 27, 24-31.

Raison, C.L., Dantzer, R., Kelley, K.W., Lawson, M.A., Woolwine, B.J., Vogt, G., … Miller, A.H. (2010). CSF concentrations of brain tryptophan and kynurenines during immune stimulation with IFN-alpha: relationship to CNS immune responses and depression. Molecular Psychiatry, 15, 393-403.

Rentesia, G., Antonioua, K., Marselosa, M., Syrroub, M., Papadopoulou-Daifotic, Z., & Konstandia, M. (2013). Early maternal deprivation-induced modifications in the neurobiological, neurochemical and behavioral profile of adult rats. Behavioural Brain Research, 244, 29–37.

Resick, P.A., Bovin, M.J., Calloway, A.L., Dick, A.M., King, M.W., Mitchell, K.S., … Wolf, E.J. (2012). A Critical Evaluation of the Complex PTSD Literature: Implications for DSM-5. Journal of Traumatic Stress, 25, 241–251.

Rich-Edwards, J.W., Spiegelman, D., Lividoti Hibert, E.N., Jun, H.J., Todd, T.J., Kawachi, I., & Wright, R.J. (2010). Abuse in childhood and adolescence as a predictor of type 2 diabetes in adult women. American Journal of Preventive Medicine, 39, 529-536.

Rinne, T., de Kloet, E.R. Wouters, L., Goekoop, J.G., de Rijk, R.H., & van den Brink, W. (2002). Hyperresponsiveness of hypothalamic-pituitary-adrenal axis to combined dexamethasone/corticotropin-releasing hormone challenge in female borderline personality disorder subjects with a history of sustained childhood abuse. Biological Psychiatry, 52, 1102-1112.

Rinne, T., de Kloet, E.R., Wouters, L., Goekoop, J.G., de Rijk, R.H., & van den Brink, W. (2003). Fluvoxamine reduces responsiveness of HPA axis in adult female

BPD patients with a history of sustained childhood abuse. Neuropsychopharmacology, 28, 126- 132.

Roelfsema, F., & Pijl, H. (2012). Phase difference between serum prolactin and cortisol rhythms is related to body mass index in humans. The Journal of Clinical Endocrinology & Metabolism, 97, E2293-E2296.

Roepke, S., Ziegenhorn, A., Kronsbein, J., Merkl, A., Bahri, S., Lange, … Lammers, C.H. (2010). Incidence of polycystic ovaries and androgen serum levels in women with borderline personality disorder. Journal of Psychiatric Research, 44, 847-852.

Roubos, E.W., Dahmen, M., Kozicz, T., Xu, L., (2012). Leptin and the hypothalamo-pituitary–adrenal stress axis. General and Comparative Endocrinology, 177, 28–36.

Runyon, M.K., Deblinger, E., & Steer, R.A. (2014). PTSD symptom cluster profiles of youth who have experienced sexual or physical abuse. Child Abuse and Neglect. 38, 84–90.

Ryu, V., Lee, J.H., Yoo, S.B., Gu, X.F., Moon, Y.W., & Jahng, J.W. (2008). Sustained hyperphagia in adolescent rats that experienced neonatal maternal separation. International Journal of Obesity, 32, 1355-1362.

Sacerdote, P., Manfredi, B., Bianchi, M., & Panerai, A.E. (1994). Intermittent but not continuous inescapable footshock stress affect immune responses and immunocyte beta-endorphin concentration in the rat. Brain, Behavior, and Immunity, 8, 251-260.

Sack, M., Sachsse, U., Overkamp, B., & Dulz B. (2013). Trauma-related disorders in patients with borderline personality disorders. Results of a multicentre study. Der Nervenarzt, 84, 608-614.

Sansone, R.A., Hahn, H.S., Dittoe, N., & Wiederman, M.W. (2011). The relationship between childhood trauma and borderline personality symptomatology in a consecutive sample of cardiac stress test patients. International Journal of Psychiatry in Clinical Practice, 15, 275-279.

Sapolsky, R.M. (2004). Mothering style and methylation. Nature Neuroscience, 7, 791-792.

Schmidt, M.V., Sterlemann, V., & Müller, M.B. (2008). Chronic stress and individual vulnerability. Annals of the New York Academy of Sciences, 1148, 174-183.

Schoedl, A.F., Costa, M.C., Mari, J.J., Mello, M.F., Tyrka, A.R., Carpenter, L.L., & Price, L.H. (2010). The clinical correlates of reported childhood sexual abuse: an association between age at trauma onset and severity of depression and PTSD in adults. Journal of Child Sexual Abuse, 19, 156-170.

Schore, A.N. (2003). Affect regulation and the repair of the self. New York: W.W Norton & Company, Inc.

Schröder, H., Ribas, L., Koebnick, C., Funtikova, A., Gomez, S.F., Fíto, M., Perez-Rodrigo, C., Serra-Majem, L. (2014). Prevalence of abdominal obesity in spanish children and adolescents. Do we need waist circumference measurements in pediatric practice? PLoS One, 9, e87549.

Schwarz, E., & Bahn, S. (2008). Biomarker discovery in psychiatric disorders. Electrophoresis, 29, 2884-2890.

Schwarz, E., Guest, P.C., Rahmoune, H., Harris, L.W., Wang, L., Leweke, F.M., ... Bahn S (2012). Identification of a biological signature for schizophrenia in serum. Molecular Psychiatry, 17, 494-502.

Seltzer, L.J., Ziegler, T., Connolly, M.J., Prososki, A,R., & Pollak, S.D. (2013). Stress-induced elevation of oxytocin in maltreated children: Evolution, neurodevelopment, and social behavior. Child Development. 85, 501-512.

Shalev, I., Moffitt, T.E., Sugden, K., Williams, B., Houts, R.M., Danese, A., … Caspi, A. (2012). Exposure to violence during childhood is associated with telomere erosion from 5 to 10 years of age: a longitudinal study. Molecular Psychiatry, 18, 576-581.

Shelton, R.C., & Miller, A.H. (2010). Eating ourselves to death (and despair): the contribution of adiposity and inflammation to depression. Progress in Neurobiology, 91, 275-299.

Shelton, R.C., & Miller, A.H. (2011). Inflammation in depression: is adiposity a cause. Dialogues in Clinical Neuroscience, 13, 41-53.

Shenk, C.E., Noll, J.G., Putnam, F.W., & Trickett, P.K. (2010). A prospective examination of the role of childhood sexual abuse and physiological asymmetry in the development of psychopathology. Child Abuse and Neglect, 34, 752-761.

Sheriff, M.J., Krebs, C.J., & Boonstra, R. Assessing stress in animal populations: Do fecal and plasma glucocorticoids tell the same story? General and Comparative Endocrinology, 166, 614–619.

Shi, Y., Devadas, S., Greeneltch, K.M., Yin, D., Allan Mufson, R., & Zhou, J.N. (2003). Stressed to death: implication of lymphocyte apoptosis for psychoneuroimmunology. Brain, Behavior, and Immunity, 17, S18-S26.

Shurin, M.R., Kusnecov, A.W., Riechman, S.E., & Rabin, B.S. (1995). Effects of a conditioned aversive stimulus on the immune response in three strains of rats. Psychoneuroendocrinology, 20, 837-849.

Smith, P., Perrin, S., Dalgleish, T., Meiser-Stedman, R., Clark, D.M., & Yule, W. (2013). Treatment of posttraumatic stress disorder in children and adolescents. Current Opinion in Psychiatry, 26, 66-72.

Smith, P., Perrin, S., Dalgleish, T., Meiser-Stedman, R., Clark, D.M., & Yule, W. (2013). Treatment of posttraumatic stress disorder in children and adolescents. Current Opinion in Psychiatry, 26, 66-72.

Spencer, S.J., & Tilbrook, A. (2011). The glucocorticoid contribution to obesity. Stress, 14, 233-246.

Steele, H. (2010). Test of time: On re-reading "Psychoanalysis and child care", John Bowlby's lecture delivered in 1956 on the centenary of Sigmund Freud's birth. Clinical Child Psychology and Psychiatry, 15, 453-458.

Sterling, P. (2004) Principles of allostasis: optimal design, predictive regulation, pathophysiology and rational therapeutics. In J. Schulkin (Ed). *Allostasis, Homeostasis, and the Costs of Physiological Adaptation* (pp. 17-63). Cambridge: Cambridge University Press.

Stone, M.H. (1993). Etiology of Borderline Personality Disorder: Psychobiological factors contributing to an underlying irritability. In J. Paris (Ed.), Borderline

Personality Disorder. Etiology and Treatment (pp. 87-101). Washington, DC: American Psychiatric Press Inc.

Suarez-Roca, H., Quintero, L., Avila, R., Medina, S., De Freitas, M., & Cárdenas, R., Central immune overactivation in the presence of reduced plasma corticosterone contributes to swim stress-induced hyperalgesia. Brain Research Bulletin, 100, 61–69.

Tang, A.C., Reeb-Sutherland, B.C., Romeo, R.D., & McEwen BS. (2013). On the causes of early life experience effects: Evaluating the role of mom. Frontiers in Neuroendocrinology, pii: S0091-3022(13)00068-X. doi: 10.1016/j.yfrne.2013.11.002

Tarullo, A., & Gunnar, M.R. (2006). Child maltreatment and the developing HPA axis. Hormones and Behaviour, 50, 632-639.

Tasca, G.A., Ritchie, K., Zachariades, F., Proulx, G., Trinneer, A., Balfour, L., … Bissada, H. (2013). Attachment insecurity mediates the relationship between childhood trauma and eating disorder psychopathology in a clinical sample: A structural equation model. Child Abuse and Neglect, 37, 926-933.

Taylor, S. (2010). Mechanisms linking early life stress to adult health outcomes. Proceedings of the National Academy of Sciences USA, 107; 8507-8512

Teicher, M.H. (2002). Scars that won't heal: the neurobiology of child abuse. Scientific American, 286, 68-75.

Teicher, M.H., Anderson, C.M., & Polcari, A. (2012). Childhood maltreatment is associated with reduced volume in the hippocampal subfields CA3, dentate

gyrus, and subiculum. Proceedings of the National Academy of Sciences USA, 109, E563-E572.

Torres, S.J., & Nowson, C.A. (2007). Relationship between stress, eating behavior, and obesity. Nutrition, 23, 887-894.

Tramantano, G., Javier, R.A., & Colon, M. (2003). Discriminating among subgroups of borderline personality disorder: an assessment of object representations. American Journal of Psychoanalysis, 63,149-175.

Trickett, P.K., Noll, J.G., Susman, E.J., Shenk, C.E., & Putnam, F.W. (2010). Attenuation of cortisol across development for victims of sexual abuse. Developmental Psychopathology, 22, 165-175.

Trifonova, S.T., Zimmer, J., Turner, J.D., & Muller, C.P. (2013). Diurnal redistribution of human lymphocytes and their temporal associations with salivary cortisol. Chronobiology International. 30, 669-681.

Tsao, C.W., & Vasan, R,S. (2013). Diagnosis: Biomarkers of cardiovascular outcomes—bonanza or bias? Nature Reviews Endocrinology, 9, 381-382.

Turnbull, A.V., & Rivier, C.L. (1999). Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. Physiological Reviews, 79, 1-71.

Turner, H.A., Finkelhor, D., & Ormrod, R. (2006). The effect of lifetime victimization on the mental health of children and adolescents. Social Science & Medicine, 62, 13-27.

Uher, R., & Weaver, I.C. (2014). Epigenetic traces of childhood maltreatment in peripheral blood: a new strategy to explore gene-environment interactions. British Journal of Psychiatry, 204, 3-5.

UNICEF South Africa. (2013). Every number tells a sad story. Retrieved from http://tinyurl.com/nywquq6.

Van den Berg, C.L., Hol, T., van Ree, J.M., Spruijt, B.M., Everts, H. & Koolhaas, J.M. (1999). Play is indispensable for an adequate development of coping with social challenges in the rat. Developmental Psychobiology, 34, 129-138.

Van der Kolk, B. (2005). Developmental Trauma Disorder. Psychiatric Annals, 35, 401-408.

Van der Kolk, B. A. (1987). The Psychological Consequences of Overwhelming Life Experiences. In B. A. van der Kolk (Ed.), *Psychological Trauma* (pp. 1 - 30). Washington DC: American Psychiatric Press, Inc.

Van der Kolk, B.A., Roth, S., Pelcovitz, D., Sunday, S. & Spinazzola, J. (2005). Disorders of extreme stress: The empirical foundation of a complex adaptation to trauma. Journal of Traumatic Stress, 18, 389-399.

Van Oers, H.J.J., de Kloet, E.R., & Levine, S. (1997). Persistent, but Paradoxical, Effects on HPA Regulation of Infants maternally deprived at different ages. Stress, 4, 249-262.

Van Oers, H.J.J., de Kloet, E.R., & Levine, S. (1998). Early vs. late maternal deprivation differentially alters the endocrine and hypothalamic responses to stress. Developmental Brain Research, 111, 245-252.

Vandell, D.L., Belsky, J., Burchinal, M., Steinberg, L., Vandergrift, N.; & NICHD Early Child Care Research Network. (2010). Do effects of early child care extend to age 15 years? Results from the NICHD study of early child care and youth development. Child Devlopment, 81, 737-756.

Veenema, A.H. (2009). Early life stress, the development of aggression and neuroendocrine and neurobiological correlates: what can we learn from animal models? Frontiers in Neuroendocrinology, 30, 497-518.

Vining, R.F., & McGinley, R.A. (1987). The measurement of hormones in saliva: possibilities and pitfalls. Journal of Steroid Biochemistry, 27, 81-94.

Vogelzangs, N., Suthers, K., Ferrucci, L., Simonsick, E.M., Ble, A., Schrager, M., … Penninx, B.W. (2007). Hypercortisolemic depression is associated with the metabolic syndrome in late-life. Psychoneuroendocrinology, 32, 151-159.

Wachs, T.D., Georgieff, M., Cusick, S., & McEwen, B.S. (2013). Issues in the timing of integrated early interventions: contributions from nutrition, neuroscience, and psychological research. Annals of the New York Academy of Sciences, 1308, 89-106.

Wajchenberg, B.L. (2000). Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocrinology Reviews, 21, 697-738.

Walsh, K., Nugent, N.R., Kotte, A., Amstadter, A.B., Wang, S., Guille, C., … Resnick, H.S. (2013). Cortisol at the emergency room rape visit as a predictor of PTSD and depression symptoms over time. Psychoneuroendocrinology, 38, 2520-2528.

Ward, G., Xing, H.C., Carnide, N., Slivchak. J., & Wainwright, P. (2004). Adrenocortical response to stress in fasted and unfasted artificially reared 12-day-old rat pups. Developmental Psychobiology, 45, 245-250.

Weaver, I.C., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., … Meaney, M.J. (2004). Epigenetic programming by maternal behavior. Nature Neuroscience, 7, 847-854.

Whittle, S., Simmons, J.G., Dennison, M., Vijayakumar, N., Schwartz, O., Yap, M.B., Sheeber, L., & Allen, N.B. (2010). Positive parenting predicts the development of adolescent brain structure: A longitudinal study. Developmental Cognitive Neuroscience, 8, 7-17.

Wingenfeld, K., Spitzer, C., Rullkötter, N., & Löwe, B. (2010). Borderline personality disorder: hypothalamus pituitary adrenal axis and findings from neuroimaging studies. Psychoneuroendocrinology, 35, 154-170

Winnicott, D.W. (1965). The family and individual development. London: Tavistock Publications Ltd.

Winnicott, D.W. (1986). Home is where we start from. Essays by a Psychoanalyst. London: Penguin Group.

Winnicott, D.W. (1987). The child, the family and the outside world. London: Penguin Group.

Wohleb, E.S., McKim, D.B., Shea, D.T., Powell, N.D., Tarr, A.J., Sheridan, J.F., & Godbout, J.P. (2013). Re-establishment of Anxiety in Stress-Sensitized Mice Is Caused by Monocyte Trafficking from the Spleen to the Brain. Biological Psychiatry, pii: S0006-3223(13)01072-X. doi: 10.1016/j.biopsych.2013.11.029.

World Health Organization. (2008). The Global Burden of Disease: 2004 Update. Switzerland, WHO Press.

World Health Organization. (2009). Global health risks. Mortality and burden of disease attributable to selected major risks. Retrieved from: http://www.who.int/healthinfo/global_burden_disease/GlobalHealthRisks_report_full.pdf

Yehuda, R., Hildebrandt, T., & Spiegel, D. (2013). Introduction to special issue of psychoneuroendocrinology "Enduring effects of traumatic stress: molecular and hormonal mechanisms". Psychoneuroendocrinology. 38, 1832-1834.

Yehuda, R., McFarlane, A.C., & Shalev, A.Y. (1998). Predicting the development of posttraumatic stress disorder from the acute response to a traumatic event. Biological Psychiatry, 44, 1305-1313.

Yehuda,R., & Seckl, J. (2011). Minireview: stress-related psychiatric disorders with low cortisol levels: a metabolic hypothesis. Endocrinology, 152, 4496-4503.

Yen, S., Gagnon, K., & Spirito, A. (2013). Borderline personality disorder in suicidal adolescents. Personal Mental Health, 7, 89-101.

Young, E., & Korszun, A. (2010). Sex, trauma, stress hormones and depression. Molecular Psychiatry, 15, 23-28.

Zanarini, M.C., Williams, A.A., Lewis, R.E., Reich, R.B., Vera, S.C., Marino, M.F., … Frankenburg, F.R. (1997). Reported pathological childhood experiences associated with the development of borderline personality disorder. American Journal of Psychiatry, 154, 1101-1106.

Zanarini, M.C., Yong, L., Frankenburg, F.R., Hennen, J., Reich, D.B., Marino, M.F., & Vujanovic, A.A. (2002). Severity of reported childhood sexual abuse and its relationship to severity of borderline psychopathology and psychosocial impairment among borderline inpatients. The Journal of Nervous and Mental Disease, 190, 381-387.

Zeanah, C.H., Berlin, L.J., & Boris, N.W. (2011). Practitioner Review: Clinical applications of attachment theory and research for infants and young children. Journal of Child Psychology and Psychiatry, 52, 819-833.

Zeeni, N., Daher, C., Fromentin, G., Tome, D., Darcel, N., & Chaumontet, C. (2013). A cafeteria diet modifies the response to chronic variable stress in rats. Stress, 16, 211- 219.

APPENDIX A

ETHICS APPROVALS

AESC 3

STRICTLY CONFIDENTIAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL ETHICS SCREENING COMMITTEE

CLEARANCE CERTIFICATE NO:

2005	74	6

APPLICANT: Dee Muller

DEPARTMENT: School of Physiology

PROJECT TITLE: The long-term physiological and behavioural effects of maternal deprivation and a subsequent aversive stressor on behavioural responses, HPA axis activation and immune response.

Species	Number	Expiry Date
Rat	251	September 2007

i) Approval is hereby given for the experiment described in the above application.

The use of these animals is subject to AESC Guidelines for the use and care of animals, is limited to the procedures specified in the application form, and to:

APPROVED subject to;

- discussing the cardiac puncture technique (i.e. who will do it), the method of euthanasia and the supply of animals with the CAS staff.

SIGNED D.A. Gray DATE: 1st October 2005

Chairman: Animal Ethics Screening Committee)

ii) I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23(1)(c) of the Veterinary and Para-veterinary Professions Act (19 of 1982)

SIGNED _____ DATE: 1st October 2005

(Registered Veterinarian)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Jacklin

CLEARANCE CERTIFICATE	PROTOCOL NUMBER M060250
PROJECT	Retrospective and Prospective Collection and Use of Demographic Statistical Data from Participants Presenting to the Clinic
INVESTIGATORS	Prof LB Jacklin
DEPARTMENT	Teddy Bear Clinic for Abused Kids
DATE CONSIDERED	06.02.24
DECISION OF THE COMMITTEE*	Approved subject to removing identifying information and using codes instead

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 06.02.27 CHAIRPERSON .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof LB Jacklin

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical))
Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

University
of the Witwatersrand,
Johannesburg

03 October 2011

Ms Rebecca Meiring
School of Physiology
Medical School
University

Dear Ms Meiring

Re: **Protocol M10635: "A Comparative Study into Bone Health of South African Pre-Pubertal Children who participate in Physical activities with Different Amounts of Skeletal Loading"**
Protocol amendment

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has reviewed and approved your request to "add an extra group of 20 children to the 2nd project" as detailed in your letter dated 04 August 2011.

Thank you for keeping us informed and updated.

Yours sincerely

Anisa Keshav
Secretary
Human Research Ethics Committee (Medical)

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5709
Private Bag 3, Wits 2050, South Africa

03 October 2011

Ms Dee Muller
School of Physiology
Medical School
University

Dear Ms Muller

RE: Protocol M070702: The Physiological Impact on Endocrine and Immune Function following Childhood Stressors"
Modification of M070724 to include plasma biomarkers measured from 20 female Children

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has reviewed and approved the following amendments on the abovementioned protocol as detailed in your letter dated 04 August 2011:

- The use of 5ml of blood from Protocol M10635
- Revised information sheet and consent form

Thank you for keeping us informed and updated.

Yours sincerely,

Anisa Keshav
Secretary
Human Research Ethics Committee (Medical)

APPENDIX B

COPYRIGHT APPROVALS

Copyright Figure 4

RightsLink | Copyright Clearance Center

Thank You For Your Order!

Dear Ms. Denise Muller,

Thank you for placing your order through Copyright Clearance Center's RightsLink service. Elsevier has partnered with RightsLink to license its content. This notice is a confirmation that your order was successful.

Your order details and publisher terms and conditions are available by clicking the link below:
http://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=38fa1919-1ba3-4e59-a2b6-aacfcdb63c76

Order Details
Licensee: Denise Muller
License Date: Jan 22, 2014
License Number: 3314200309821
Publication: Epilepsy & Behavior
Title: Early-life stress and HPA axis trigger recurrent adulthood depression
Type Of Use: reuse in a thesis/dissertation
Total: 0.00 USD

To access your account, please visit https://myaccount.copyright.com.

Please note: Online payments are charged immediately after order confirmation; invoices are issued daily and are payable immediately upon receipt.

To ensure we are continuously improving our services, please take a moment to complete our customer satisfaction survey.

B.1:v4.2

+1-877-622-5543 / Tel: +1-978-646-2777



Copyright Figure 1



Thank You For Your Order!

Dear Ms. Denise Muller,

Thank you for placing your order through Copyright Clearance Center's RightsLink service. Nature Publishing Group has partnered with RightsLink to license its content. This notice is a confirmation that your order was successful.

Your order details and publisher terms and conditions are available by clicking the link below:
http://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=09ebc9b7-3173-41b7-af20-2ad28cee5075

Order Details
Licensee: Denise Muller
License Date: Jan 22, 2014
License Number: 3314221442925
Publication: Nature Neuroscience
Title: How adversity gets under the skin
Type Of Use: reuse in a dissertation / thesis
Total: 0.00 USD

To access your account, please visit https://myaccount.copyright.com.

Please note: Online payments are charged immediately after order confirmation; invoices are issued daily and are payable immediately upon receipt.

To ensure we are continuously improving our services, please take a moment to complete our customer satisfaction survey.

B.1:v4.2

Author Copyright Figure 1

Fine with me.

Steve Hyman

On Tue, Sep 17, 2013 at 10:10 AM, Dee Muller <Dee.Muller@wits.ac.za> wrote:

Hi Professor Hyman

I would like to request permission to use Figure 2 (with full acknowledgement of the source)from your New and Views paper entitled ” How adversity gets under the skin” Nature Neuroscience, 2009, 12, 241-243 in my PhD thesis.

Thanking you

Ms Denise Muller

School of Physiology

University of Witwatersrand

South Africa

Protective and damaging effects of stress mediators: central role of the brain

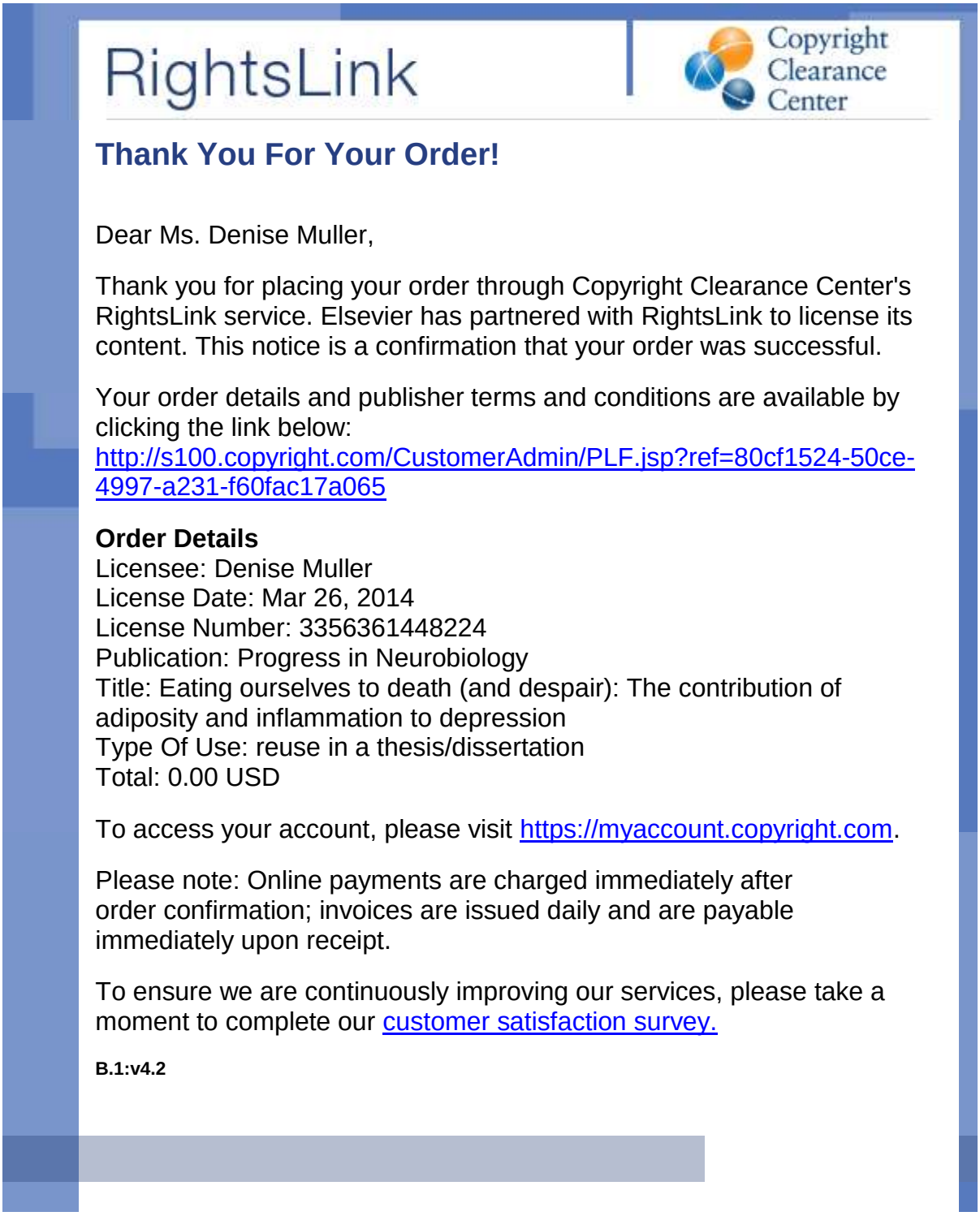
Bruce S. McEwen, PhD*

Author information ► Copyright and License information ▼

Copyright : © 2006 LLS

This is an open-access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by-nc-nd/3.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Copyright Figure 6



RightsLink

Copyright Clearance Center

Thank You For Your Order!

Dear Ms. Denise Muller,

Thank you for placing your order through Copyright Clearance Center's RightsLink service. Elsevier has partnered with RightsLink to license its content. This notice is a confirmation that your order was successful.

Your order details and publisher terms and conditions are available by clicking the link below:
http://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=80cf1524-50ce-4997-a231-f60fac17a065

Order Details
Licensee: Denise Muller
License Date: Mar 26, 2014
License Number: 3356361448224
Publication: Progress in Neurobiology
Title: Eating ourselves to death (and despair): The contribution of adiposity and inflammation to depression
Type Of Use: reuse in a thesis/dissertation
Total: 0.00 USD

To access your account, please visit https://myaccount.copyright.com.

Please note: Online payments are charged immediately after order confirmation; invoices are issued daily and are payable immediately upon receipt.

To ensure we are continuously improving our services, please take a moment to complete our customer satisfaction survey.

B.1:v4.2