# Mechanisms in social anxiety disorder in the context of early developmental trauma: An imaging, neurocognitive and genetics study

by

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## DECLARATION

I, **David Rosenstein**, the undersigned hereby declare that the work contained in this

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**"I wondered how many people there were in the world who suffered, and continued to suffer, because they could not break out from their own web of shyness and reserve, and in their blindness and folly built up a great distorted wall in front of them that hid the truth."**

— Daphne du Maurier, *Rebecca*

**"A stammering man is never a worthless one. Physiology can tell you why. It is an excess of sensibility to the presence of his fellow creature, that makes him stammer."**

— Thomas Carlyle, letter to Ralph Waldo Emerson, 1843 November 17th

# SUMMARY

The contribution of early developmental trauma (EDT) to the neurobiological mechanisms of social anxiety disorder (SAD) is poorly understood. A cross sectional study was conducted to examine the effects of EDT on the pathophysiological mechanisms in SAD. The study assessed three groups: SAD with EDT, SAD without EDT and healthy controls matched for age, gender, ethnicity, education and handedness. We conducted neurocognitive testing, DNA sequencing from whole blood, structural magnetic resonance imaging and proton magnetic resonance imaging. Bayesian statistical analyses were conducted, as the philosophical and theoretical underpinnings of Bayesian statistical methods account for many of the limitations inherent in traditional statistical methods. Bayesian statistical analysis was also used due to the limitations presented by the data in this study. The SAD with EDT group had a smaller left caudate nucleus compared with the SAD without EDT group and smaller left and right anterior cingulate cortex and left thalamus compared with controls. The SAD with EDT group had higher inositol (Ins) and lower N-Acetylaspartate (NAA) concentrations in the amygdala compared with controls, and higher concentrations of Glutamine (Gln) and Glutamate (Glu) compared with controls. In contrast, the SAD without EDT group only had higher concentrations of Glu and Gln relative to controls. Further the SAD with EDT group had neurocognitive difficulties compared with controls spanning domains such as verbal immediate and delayed memory recall, executive functioning and fine motor functioning. The SAD without EDT group only demonstrated difficulties of immediate and delayed verbal memory compared with controls. Heterozygous (*TG*) carriers of the *TPH2* gene had higher total social anxiety scores and higher scores on the social fear and avoidance subscales than homozygous (*GG*) carriers. In addition individuals who carried at least one *C* allele of the *RGS2* gene, compared with homozygous *GG* carriers, had higher concentrations of NAA and Ins; both metabolites are indicative of neuronal integrity. A number of correlations were observed between neurocognitive test findings and specific structural grey matter volumetric abnormalities, mainly in the SAD with EDT group. In the SAD with EDT group fine motor dexterity in the dominant hand was negatively correlated with the left caudate nucleus. In the SAD with EDT group executive functioning represented by non-perseverative errors negatively correlated with the right amygdala and conceptual level responses correlated positively with the right amygdala. No significant correlations were found in fine motor

performance in the non-dominant hand and grey matter volumes. In the SAD without EDT group immediate memory recall was positively correlated with the left ACC and delayed verbal memory was positively correlated with the right ACC. In conclusion this study provides novel insights into the mechanisms of SAD within the context of EDT compared with SAD without EDT and healthy controls. A number of limbic circuitry volume differences, neurocognitive performance difficulties and more profuse dysregulated neurometabolism of the left amygdala characterizes individuals with SAD and EDT compared to individuals with SAD without EDT, demonstrating the additional influence of EDT on the pathophysiology of SAD.

# OPSOMMING

Vroeë Ontwikkelings Trauma (VOT) se bydrae tot die neurobiologiese meganismes van Sosiale Angs Versteuring (SAV) is onduidelik. 'n Deursnit studie is gedoen om die effek van VOT op die patofisiologiese meganismes van SAV te ondersoek. Drie groepe is ondersoek: SAV met VOT, SAV sonder VOT en 'n gesonde kontrole groep, wat ooreenstem met die ondersoek groepe op grond van ouderdom, geslag, etnisiteit, opleiding en hand voorkeur. Neurokognitiewe toetse, deoksiribonukleïensuur (DNS) volgorde bepaling vanuit heelbloed, strukturele magnetiese resonans beelding en proton magnetiese resonans beelding is gedoen. Bayes statistiese analise is gedoen, omdat die filosofiese en teoretiese grondslag van Bayes se statistiese metodes baie van die beperkings van tradisionele statistiese metodes in ag neem. Dit is ook gebruik as gevolg van die beperkings wat die data van hierdie spesifieke studie te vore gebring het. Die SAV met VOT groep het 'n kleiner linker kaudaat kern in vergelyking met die SAV sonder VOT groep en 'n kleiner linker en regter anterior singulaat korteks (ASK) en linker talamus wanneer hulle met die kontrole groep vergelyk word. Die SAV met VOT groep het ook laer Inositol (Ins) en N-Asetielaspartaat (NAA) vlakke en hoër konsentrasies van Glutamien (Gln) en Glutamaat (Glu) in die amigdala as hulle vergelyk word met die kontrole groep. In teenstelling daarmee het die SAV sonder VOT groep slegs hoër konsentrasies van Gln en Glu getoon relatief tot die kontrole groep. Verder het die SAV met VOT groep neurokognitiewe disfunksie in verbale onmiddellike en vertraagde geheue herroeping, uitvoerende funksionering en fyn motor funksionering in vergelyking met die kontrole groep. Die SAV sonder VOT groep het slegs gebreke in onmiddellike en vertraagde geheue herroeping getoon in vergelyking met die kontrole groep. Heterosigotiese (*TG*) draers van die *TPH2* geen het hoër totale sosiale angs tellings en hoër tellings op sosiale vrees en vermyding subskale as homosigotiese (*GG*) draers. Daar is ook gevind dat individue wat ten minste een *C* alleel dra van die *RGS2* geen, wanneer hulle vergelyk word met homosigotiese *GG* draers, hoër vlakke NAA en Ins het. Altwee hierdie metaboliete gee 'n aanduiding van neuronale integriteit. Verskeie korrelasies is gevind tussen neurokognitiewe toets bevindings en spesifieke grysstof volumetriese abnormaliteite, waarvan die meeste in die SAV met VOT groep is. In die SAV met VOT groep het fyn motor vaardigheid met die dominante hand negatief korreleer met die linker kaudaat kern. In die SAV met VOT groep het uitvoerende funksionering, verteenwoordig deur nie-aanhoudende foute, negatief

korreleer met die regter amigdala en begrips vlak antwoorde positief korreleer met die regter amigdala. Geen betekenisvolle korrelasies is gevind in fyn motor werkverrigting met die non-dominante hand en grysstof volumes nie.  In die SAV sonder VOT groep het onmiddellike geheue herroeping positief korreleer met die linker ASK en vertraagde verbale geheue het positief korreleer met die regter ASK.  Ten slotte, die studie het nuwe bevindinge voorsien in die meganismes van SAV in die konteks van VOT in vergelyking met SAV sonder VOT en gesonde kontroles. Individue met SAV en VOT word gekarakteriseer deur verskeie limbiese baanwerk volume verskille, neurokognitiewe werksverrigting moeilikhede en erger ontwrigte neurometabolisme in die linker amigdala as hulle vergelyk word met individue met SAV sonder VOT.  Dit demonstreer die bykomende effek van VOT op die patofisiologie van SAV.

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8

# PUBLICATIONS AND PRESENTATIONS BASED ON THIS WORK

**1)** Bishop, M., **Rosenstein, D**., Bakelaar, S. and Seedat, S. (2015). An analysis of early developmental trauma in social anxiety disorder and posttraumatic stress disorder. *Annals of General Psychiatry*, 13(16) http://www.annals-general-psychiatry.com/content/13/1/16


**2**) **Rosenstein, D.**, Zwart, J., Bakelaar, S., Bishop, S. and Seedat, S. (Currently in review). Neurocognitive deficits in social anxiety disorder in the context of early developmental trauma: A Bayesian analysis. *BMC Psychaitry*. **(Currently in Review).**

**3)** **Rosenstein, D**., Hess, A., Zwart, J., Ahmed-Leito, F., Meintjies, E. and Seedat, S. (Currently in review). Single voxel proton magnetic resonance spectroscopy (^{1}H-MRS) of the amygdala in social anxiety disorder in the context of early developmental trauma. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*. **(Currently in Review).**

**4)** **Rosenstein, D.,** Ahmed-Leitao, F., Fouche, J.P., Zwart, J. and Seedat, S. (to be submitted). Structural magnetic resonance imaging (sMRI) in social anxiety disorder in the context of early developmental trauma: a Bayesian analysis.

# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| **ACC** | – | Anterior Cingulate Cortex |
| **BI** | – | Behavioral Inhibition |
| **CBT** | – | Cognitive Behavior Therapy |
| **DSM IV** | – | Diagnostic and Statistical Manual of mental disorders 4$^{th}$ edition |
| **DSM 5** | – | Diagnostic and Statistical Manual of mental disorders 5$^{th}$ edition |
| **EDT** | – | Early Developmental Trauma |
| **fMRI** | – | Functional Magnetic Resonance Imaging |
| **Gln** | – | Glutamine |
| **Glu** | – | Glutamate |
| **Glx** | – | Glutamate/Glutamine |
| **GPB** | – | Grooved Peg Board |
| **$^1$H** | – | Protons/Hydrogen |
| **$^1$H-MRS** | – | Proton Magnetic Resonance Spectroscopy |
| **HVLT** | – | Hopkins Verbal Learning Test |
| **Ins** | – | Inositol |
| **MCMC** | – | Marcov Chain Monte Carlo |
| **MRI** | – | Magnetic Resonance Imaging |
| **NAA** | – | N-Acetylaspartate |
| **NHST** | – | Null Hypothesis Significance Testing |
| **PCr** | – | Phosphocreatine |
| **PTSD** | – | Post Traumatic Stress Disorder |
| **RGS2** | – | Regulator of G-protein signaling (RGS2) |
| **SAD** | – | Social Anxiety Disorder |
| **Stroop** | – | Stroop Tests |
| **sMRI** | – | Structural Magnetic Resonance Imaging |
| **TPH2** | – | Tryptophan hydroxylase |
| **WCST** | – | Wisconsin Card Sorting Test |
| **WMS** | – | Wechsler Memory Scales |

## LIST OF FIGURES                                                    PAGE

**LIST OF TABLES** **PAGE**

# CONTENTS                                                      PAGE

# 1. INTRODUCTION

**The introductory chapter provides the rationale and significance for the study. The aims, objectives and hypotheses follow from the introduction. Lastly an overview of the various chapters in this dissertation will be presented.**

## 1.1  *Introduction and significance*

Early developmental trauma is common to both social anxiety disorder (SAD) and posttraumatic stress disorder (PTSD). Neurocognitive studies in SAD and PTSD have documented various deficits, including verbal learning and memory (Asmundson et al., 1994) and executive function (Etkin & Wager, 2007) in both disorders. Moreover, functional imaging studies have found exaggerated amygdala and insula activation to emotional faces in both disorders; notably, with more pronounced exaggeration in these regions observed in SAD than in PTSD, and some studies have reported decreased rather than increased amygdala activation in PTSD (Etkin and Wager, 2007). Structural magnetic resonance imaging investigations (sMRI) have also found grey matter abnormalities and morphometric differences in individuals with SAD compared with controls (Potts et al, 1994; Talati, Pantazatos, Schneier, Weissman & Hirsch, 2013; Talati, Pantazatos, Hirsch, & Schneiera, 2015), however studies are few. Neurometabolite changes have also been observed, notably in the glutamate (Glu) signal and Glu receptors, however regional neurometabolism in the amygdala in SAD has not been extensively studied. The extent to which neurometabolite changes may be mediated by early developmental trauma (EDT) in adults with SAD has not been clearly elucidated and, as such, is the focus of this study. Psychological trauma is a very present aspect of South African communities, given the high crime rates, social transition, economic difficulties and our past political injustices. Such trauma often occurs at an early stage of life and may express itself in neural centers involved in interpersonal emotive processing, such as the amygdala. If EDT does increase an individual's propensity to develop SAD, it is important to understand the neurobiological mechanisms involved, as this may have implications for treatment of the disorder and its potential early prevention.

1.2   *Objectives*

1.2.1    Primary objectives

(i) To determine whether individuals with generalized SAD and early developmental trauma have significantly smaller grey matter cortical and limbic (i.e. amygdala, hippocampus) volumes compared with individuals with generalized SAD without early developmental trauma and healthy controls (matched on age, gender, sex, educational level, neurological and psychiatric history including substance abuse history)

(ii) To determine whether individuals with generalized SAD and early developmental trauma have dysregulated neurometabolism in the amygdala, approximating what has been found in PTSD and anxiety disorder literature in the amygdala, compared with individuals with generalized SAD without early developmental trauma and healthy controls;

(iii) To investigate the predictive power of specific genotypes and behavioural measures of anxiety on amygdala volumes and neurometabolism across the 3 groups.

1.2.2 *Secondary objectives*

(i)  To examine verbal learning difficulties in individuals with SAD with EDT and compare the, to individuals who are socially anxious without EDT, and to healthy controls.

(ii)  To examine memory function difficulties in individuals with SAD with EDT and compare them to individuals who are socially anxious without EDT, and to healthy controls.

(iii)  To examine executive functioning difficulties in individuals with SAD with EDT and compare them to individuals who are socially anxious without early developmental trauma, and to healthy controls.

(iv)  Lastly to compare the neurocognitive test findings with magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopic (^{1}H-MRS) analysis of individuals with SAD and early developmental trauma and compare these findings across the 3 groups (SAD with EDT, SAD without EDT and healthy controls).

1.3   *Hypotheses*

This study hypothesized that socially anxious individuals with EDT would show (i) significantly greater reductions in grey matter volume in cortical and limbic regions (approximating what has previously been observed in patients with PTSD), than those without EDT; (ii) significantly greater neurometabolite abnormalities within the amygdala, a limbic structure involved in anxiety disorder aetiology (approximating what has previously been observed in patients with PTSD), than those without EDT; and (iii) more pronounced

19

neurocognitive difficulties in neurocognitive test performance (e.g. verbal memory, fine motor functioning and executive function) than those without EDT and relative to healthy controls. The study additionally hypothesized that (i) the aforementioned neurocognitive performance difficulties in individuals with SAD with EDT would correspond with region-specific MRI volume shifts and dysregulated neurometabolism in the amygdala; and (ii) within the two SAD groups and the control group, we expected carriers of high-response variants of TPH2 and *RGS2* genotypes to show more abnormal amygdala reactivity relative to the low-response variant carriers in terms of dysregulated neurometabolism.

### *1.4   Overview of chapters*

***Chapter 1*** provides an overview of the thesis and first lays out the rationale for conducting the study and discusses the hypotheses and objectives.

***Chapter 2*** provides an overview of social anxiety disorder within the context of EDT. It outlines the literature on neurocognition, genetics and neuroimaging both in SAD and in EDT.

***Chapter 3*** outlines the methods used in the study. This includes the study design, sampling and participant selection, instruments, neuroimaging parameters, genetic sequencing, and statistical techniques and data analysis.

*The following chapters discuss the various findings of the research. Each chapter presents a comprehensive account of findings related to specific mechanisms of social anxiety within the context of early developmental trauma. These chapters are also presented in a journal paper format, some of these chapters are in the process of being published or have been published:*

***Chapter 4*** details the neurocognitive performance difficulties found between the groups. This chapter mainly presents neurocognitive findings in the domains of memory, executive functioning and fine motor performance. Data is presented predominantly within a Bayesian framework (manuscript under review).

***Chapter 5*** details the sMRI findings of the various grey matter volume differences between the groups (manuscript in preparation).

*Chapter 6* provides the ^{1}H-MRS findings of the neuromtabolite differences across the three groups under investigation. This chapter provides evidence for functional processing of the amygdala in SAD within the context of EDT (article under review).

*Chapter 7* provides correlational data between neurocognitive and sMRI findings, and examines genotypic associations.

*Chapter 8* is the discussion, which brings the various components of the study of the mechanisms of SAD in the context of EDT together. It first examines neurocognition and structural/ morphometry findings. It then discusses amygdala functioning and lastly examines the genetics data in relation to the sMRI data.

*Chapter 9* presents the conclusions and recommendations for future research.

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# 2. BACKGROUND

**The background chapter introduces the pertinent literature on social anxiety disorder (SAD), its current clinical status and epidemiology. It will then define and discuss early developmental trauma (EDT). A succinct summary of the mechanisms of SAD will be provided. These mechanisms will each in turn be discussed within the context of EDT.**

2.1 *An overview of social anxiety disorder (SAD)*

Hippocrates first described social fears and in the 1870s they were systematically delineated from other phobias (Marks, 1970, 1985; McNeil, 2014). The diagnostic nosology of Social Anxiety Disorder (SAD) was first conceptualized within the diagnostic framework of the DSM nomenclature in the 1980's in the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III). In this iteration of the DSM, social phobia was considered as a subdivision of the phobic disorders along with agoraphobia and the specific phobias (Hofmann, 2010; DSM-III, APA 1980). However since the early 1900's, the term 'social phobia' has been used to describe individuals with performance anxiety and has been loosely used to define characteristic phobic reactions and behaviors to social interactions and situations (Berman & Schneier, 2004). Specific diagnostic criteria for SAD began to emerge in the DSM-III as researchers noticed that social anxiety disorder or social phobia differed from the other phobias in terms of age of onset and course (Marks & Gelder, 1966 cited in Berman & Schneier, 2004).

In some of the very first studies of SAD and the phobias in the 1960's and 1970's, Marks and Gelder (1966) made some distinct categorizations regarding social phobia. These included component fears such as: "fears of eating, drinking, shaking, blushing, speaking, writing, or vomiting in the presence of other people," with the most fundamental feature being a fear of appearing foolish to others or embarrassed, thus leading to marked avoidance (McNeil, 2014; Marks & Gelder, 1966). Over the ensuing years, between DSM-III and the development of DSM–IV, SAD received little research attention (McNeil, 2014; Marks & Gelder, 1966). The scientific language has many different terms that apply or relate to SAD, including: shyness, social anxiety, social withdrawal, social phobia, behavioral inhibition (BI), communication apprehension, and introversion. These various states have been described in a myriad of

23

different ways (McNeil, 2014), however they include all component aspects of SAD (McNeil, 2014).

The term social phobia has been suggested as being misleading, and there have been recommendations that it should be discarded, as avoidance of specific circumscribed phenomena, object or activity is at the heart of the fear or anxiety in SAD (Bögels & Stein, 2009). This is especially pertinent as individuals who suffer from SAD find it hard to avoid social situations and often have to face them due to social pressures, and a desire to be social, and interactions and social situations are hard to avoid because they are ubiquitous (Bögels & Stein, 2009).

SAD has been retained as a diagnostic entity in the current fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5, 2013). Current DSM-5 nomenclature characterizes SAD as "a marked and persistent fear of social or performance situations in which embarrassment may occur. Exposure to the social or performance situation almost invariably provokes an immediate anxiety response" (DSM-5, APA 2013). At current the DSM-5 presents the most accurate description of the presentation of the disorder (McNeil, 2014).

### 2.1.1   SAD subtypes

Although DSM-IV-TR (APA, 2000) does not refer to any residual categories of SAD, SAD is divided into two subtypes, the *specific* and *generalized* subtypes (Aderka, Nickerson & Hofmanna, 2012). These subtypes appear to be qualitatively the same, yet are quantitatively different, lying on a spectrum of severity (see Figure 1), with the specific subtype of SAD being less severe and the generalized subtype more severe in symptom presentation, subjective distress and level of psychological and social impairment (Aderka et al, 2012).

low fear ➡ 'normal' fear ➡ shyness ➡ specific SAD ➡ generalised SAD ➡ avoidant PD
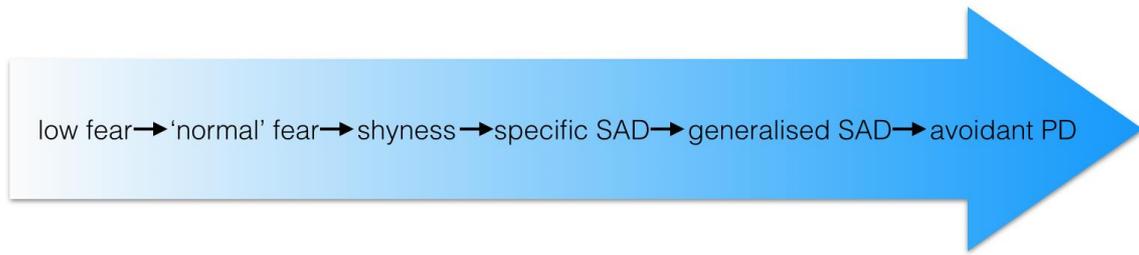
Figure 1 illustrates SAD as conceptualized as lying on a spectrum with shyness and non-pathological forms on one end and extreme social anxiety and avoidant personality disorder (Avoidant PD) on the other.

There has been a suggestion that subtypes of SAD should be distinguished on the content of fearful situations, and the DSM-5 work group suggested that SAD may be subtyped according to performance situations and social situations (Bögels & Stein, 2009; Bögels et al, 2010; McNeil, 2014).

Research on SAD subtypes has demonstrated that the generalized and specific subtypes falls short of adequately denoting the clinical presentation of SAD (Knappe, Beesdo-Baum, Fehm, Stein, Lieb, Wittchen, 2011). Knappe et al. (2011) found that individuals with SAD often have more than one situational or interactional fear and that the generalized and specific subtypes do not fully represent the clinical expression of these fears or the etiological origins of SAD (Knappe, et al., 2011).

An admixture analysis of the current generalized and specific SAD subtypes revealed that both subtypes belong to the same underlying population of origin. In the same study it was observed that differences in comorbidity such as depression and generalized anxiety were not significant when social anxiety severity was controlled for (Aderka, Nickerson & Hofmann, 2012).

In summary, the current view of SAD is a disorder that lies on a single continuum, rather than as a disorder with subtype categorical constructs (viz., generalized and specific SAD) (El-Gabalawy, Cox, Clara & MacKenzie, 2010).

*2.1.2   DSM-5 changes to SAD*

One of the main thrusts in the development of the DSM-5 was to examine psychopathology as spectrum disorders rather than discrete conditions (Bögels, Alden, Beidel, Clark, Pine, Stein and Voncken, 2010). With regards to SAD the debate appeared to be focused predominantly on the subtypes and whether or not they represent an SAD spectrum or whether they are independent constructs with regards to aetiology, symptom presentation and underlying neurobiology (Bögels, et al, 2010; Aderka et al, 2012).

There has been relatively little investigation of the various presentations and clinical specifiers in SAD (Bögels, et al, 2010). For example the 'generalized' subtype specifier in DSM-IV suggests that an individual fears "most social situations" (DSM-IV, 1994), however there is no clarification with regards to what this means. The literature suggests disparate recommendations with regards to what the 'generalized' subtype may delineate (Bögels, et al, 2010). Researchers have operationalized the generalized subtype in different ways, from the number of social feared situations to the types of situations feared. However there has been little consistency with regards to the factors and content that defines "most social situations" (Bögels, et al, 2010).

According to Bögels, et al (2010) the DSM-5 Task Force raised specific questions regarding the diagnostic presentation of SAD, these included: (1) What evidence is there for the DSM-IV generalized sub-type specifier? If there is insufficient evidence for this specifier, does any other evidence exist for other specific specifiers based on the content of feared situations? (2) Is there evidence for showing/displaying symptoms of anxiety and is fearing the display of symptoms an aspect of SAD? (3) Is test anxiety an aspect of SAD? (4) Is selective mutism a symptom or subtype of SAD? (5) Is there evidence to support avoidant personality disorder as a subtype of SAD? (6) Is SAD a valid diagnosis for children and adolescents and what is the earliest age of onset?

The only predominant departure DSM-5 made to the diagnostic specifiers in SAD was the definitional change from "the duration is at least 6 months" (DSM IV-TR, 1994) to "the fear, anxiety, or avoidance is persistent, typically lasting 6 or more months."  The rest of the diagnostic specifiers remain the same.  SAD can be conceptualized as lying on a spectrum with shyness and non-pathological forms on the one end and extreme social anxiety and

avoidant personality disorder on the other (see Figure 1), however there are still two subtypes according to current diagnostic nomenclature (APA, 2013).

### 2.1.3    *Epidemiology of SAD*

Early epidemiological surveys for SAD, such as the Epidemiological Catchment Area Study (ECA) (Regier et al. 1993) and the National Comorbidity Survey (NCS) (Kessler et al. 1999), found SAD to be one of the most common psychiatric disorders and the most common anxiety disorder. Cross-culturally SAD is considered to be the third most common mental illness and the most common anxiety disorder (Hofmann, 2010). More recent epidemiological surveys have found (NESARC, Grant et al. 2005) a lifetime prevalence of approximately 5% compared with the 13% documented in the original NCS study (Grant et al., 2005). According to the nationally representative SASH study (Herman, Stein, Seedat, Heeringa, Moomal & Williams, 2009, the most prevalent class of lifetime disorders among South Africans are anxiety disorders (15.8%), with the lifetime prevalence of SAD at 2.8%. Vriends, Bolt and Kunz (2014) in a review of summarizes of prospective and retrospective spontaneous remission rates of SAD published after 1987 found remission rates of SAD varied between 36% and 93% and in retrospective studies between 3% and 80%. The estimated average remission rate in prospective studies was 50% for full remission and 79% when including partial remission. In retrospective studies, the average remission rate was 26% and 56% over the lifetime. Diverse predictors of remission of SAD have been found that can be clustered into less severe anxiety, less additional psychopathology, less stress, and being employed (Vriends, et al., 2014).

Anxiety disorders are generally more prevalent in females than males (Bögels, et al, 2010; McLean, et al, 2011; Baxter, et al, 2013). SAD usually begins in early childhood and adolescence, follows a chronic course and can be highly debilitating in terms of academic, work, and social functioning (Schneier et al. 1994; Detweiler, Comer, Crum & Albano, 2014). The age of onset for SAD is usually between 12 and 13 years, however some evidence suggests that it may be earlier than this (Bandelow & Stein, 2005). There is also compelling evidence that SAD has strong childhood developmental origins (Ollendick, Hirshfield & Becker, 2002; Lahat, et al. 2014; Bruce, et al., 2013; Detweiler, Comer, Crum & Albano, 2014). Disruption in social emotional developmental circuitry may result from dysfunctional modeling or EDT (Heim, et al. 2013; Whalley, 2013; Sheu et al, 2014).

*2.2  Early developmental trauma (EDT)*

Throughout development, the experience of stressors is both important and beneficial, for learning and adaptation and for the appropriate development of biological stress response systems (Cozolino, 2006). However, prolonged, maladaptive or overwhelming stressors in the form of EDT's may negatively affect the development of a growing child's stress response systems, especially of the brain and specifically the amygdala (Cozolino, 2006). Understanding the impact of these EDT's may constitute a critical next step in understanding the pathophysiological mechanisms of SAD. EDT may be defined as a single or multiple traumatic experience(s) that a child experiences up until the age of 18 years. This study examined EDT within the framework of the Childhood Trauma Questionnaire (CTQ), which examines five categories of EDT (Bernstein & Fink, 1998; Bernstein et al., 2003), which include: physical abuse (PA), emotional abuse (EA), emotional neglect (EN), physical neglect (PN) and sexual abuse (SA) (Bernstein, Ahluvalia, Pogge, & Handelsman, 1997).

EDT is believed to significantly influence the developing emotional systems of the brain (Holmes et al., 2005; Marusak, Etkin & Thomason, 2015). The impact may vary from significant changes in neurological organization of structural neural networks of emotional processing centers, to changes in neurotransmission and neuro-hormone modulation. For example, Holmes, et al. (2005) found that early maternal separation in rodents significantly affects neurotransmitter systems implicated in emotional processing; corticotropin-releasing factor, serotonin, norepinephrine, and glutamate processing and production. Neural organization of emotion may be divided functionally, and to a large extent structurally, into primary and secondary emotional systems (Zillmer & Spiers, 2001), with emotional processing varying according to the type of emotion. Various neural subsystems process specific emotions with each subsystem ultimately integrated with the limbic system (Zillmer & Spiers, 2001).  Limbic processing of fear may shortcut higher cortical processing, so that the amygdala processes information relating to fear by directly bypassing the cortical loop and thalamic-cortical projections (Zillmer & Spiers, 2001).

EDT may loosely be defined as any traumatic experience that occurs before 18 years of age (Bernstein, 1998). It has been estimated that, on average, one in ten children annually is neglected or psychologically abused and that approximately 4%-16% have been physically

28

abused (Gilbert et al, 2008). These early developmental traumas constitute a significant aetiological risk in the development of psychopathology during childhood and in later adult life.

EDT is also known to increase the propensity for emotional disorders (Holmes et al., 2005). Early childhood trauma may not only be a risk factor for the development of emotional disorders, but may also contribute to the earlier onset, increased comorbidity, and the decreased efficacy of treatments (Brodsky et al., 2001; Friedman et al., 2002; Gladstone et al., 1999, 2004; Matza et al., 2003; McHolm et al., 2003; Nemeroff et al., 2003; Romans et al., 1995; Zlotnick et al., 1994). Heim and Nemeroff (2001) have suggested a strong link between adult anxiety disorders and early childhood trauma. They suggest that children who have experienced early trauma may be strongly predisposed to develop later anxiety disorders, such as obsessive compulsive disorder (OCD), panic disorder, PTSD and SAD (Heim & Nemeroff, 2001).

EDT significantly affects one's propensity for developing a psychiatric condition later in life, even when controlling for the characteristics of the individual, the social environment, and the specifics of the traumatic episode (Pine & Cohen, 2002). EDT compared with other childhood aversive life difficulties may be more salient in the development of later life anxiety disorders (Hovens et al. 2010). A number of studies have examined the effects of childhood trauma, in comparison with early aversive life events, and found that childhood trauma had the most significant effect on the development of later life psychopathology; specifically with regards to the later development of anxiety and affective disorders (Spinhoven et al., 2010). Heim et al. (2008; Heim & Nemeroff, 2002) and Bradley et al. (2008) found that early developmental trauma affects the regulation of the HPA-axis and the severity of psychopathology in a subgroup of depressed patients. The duration of the course of distress and psychopathology is also mediated by early developmental trauma (Landrigan et al., 2008), with the course of the disorder usually longer in the face of early developmental trauma (Landrigan et al., 2008) Matz, Junghöfer Weber,Wienbruch & Rockstroh (2010).

The effect of EDT on physiological and psychological functions and wellbeing has been verified in a number of animal studies (Plotsky et al., 2005; Spinelli et al., 2009). Individuals suffering from EDT also display abnormal cortical activation when shown affective pictorial stimuli (Weber, et al., 2009). This was reflected in a dampening of the cortical response of

29

individuals with EDT compared with controls in one study (Weber et al., 2009). Childhood adversity and trauma contribute to the development of the cortical processing of stress and specifically increased stress sensitivity (Matz, Junghöfer Weber,Wienbruch & Rockstroh, 2010; Marusak et al, 2015).

EDT may present in a number of forms. In its milder but pervasive form, it may occur in the context of poor parenting or rearing styles. Parental neglect and other traumatic experiences in childhood may manifest as a more severe and chronic form of EDT. It may also occur in the context of familial mental disorders or birth risk factors (Bandelow et al., 2004). With respect to SAD, family factors have also been considered to be strongly associated with the pathogenesis of SAD; these factors may include child-rearing styles, restricted exposure to social situations in the family, and parental modeling of early developmental trauma and traumatic conditioning experiences (Bandelow et al., 2004; Blanco et al., 2004; Bandelow, Torrente & Ruther, 2004). Most studies of EDT are based on retrospective data and may, therefore, be biased with regards to distortions in memory recall (Bandelow et al., 2004).

High rates of EDT are consistently present in many cases of SAD (Bandelow et al., 2004). Bandelow, et al. (2004) in a sample of 50 patients with SAD, using a 203 itemized 'severe trauma scale', found a significant relationship between EDT and SAD. EDT was reported significantly more frequently in individuals with SAD than in healthy controls (Bandelow, et al., 2004). Individuals with SAD reported severe traumatic experiences, while none were reported in healthy controls. In another study, Stein et al. (1996) examined childhood physical and sexual abuse in 125 patients with anxiety disorders (panic disorder, social phobia and obsessive-compulsive disorder) compared with healthy controls and found higher rates of abuse in patients with anxiety disorders, however results were not reported separately for the 55 patients with social phobia relative to the patients with other anxiety disorders (Stein et al., 1996).  Chartier et al. (2001) examined a non- clinical sample identified as having social phobia and found high rates of EDT experiences. Tweed et al (1989) found, in a non-clinical sample, no significant association between social anxiety disorder and parental death or separation; however, other types of EDT were not investigated.

Anxiety disorders have been found to be significantly more common than mood disorders in children who have experienced EDT (Ackerman, Newton, McPherson, Jones, & Dykman, 1998). Exposure to serious adverse early events in childhood, apart from EDTs, is a risk

factor for later life psychiatric symptoms; this holds true even when controlling for other characteristics of the individual such as the social environment and the nature of the traumatic event (Pine & Cohen, 2002). However EDT may be more salient in the development of anxiety disorders later in life than childhood aversive life events (Hovens et al. 2010). Hovens et al (2010) compared childhood life events which included parental divorce, early parental loss and 'placed in care' and EDT which included PA, EA, SA and EN, prior to the age of 16 and found EDT rather than childhood aversive events contributed more greatly to later onset of anxiety disorders. This has also been found in a similar study by Spinhoven et al (2010), where the researchers examined the association between childhood adversities and negative life experiences across the lifespan in individuals with a lifetime-based diagnosis of depression and/or anxiety disorder. They controlled for comorbidity and the clustering of adversities and found childhood adversities had a stronger association with affective disorders than negative life events across the lifespan (Spinhoven, et al, 2010).

Human developmental is vulnerable to abnormal, extreme and repetitive patterns of stress during critical periods in brain development (Anda et al, 2006). Since the developing brain is not equipped with a mature stress response and regulatory ability (Anda et al, 2006) the consequences of persistent stress could be potentially damaging. Extremely stressful experiences can impair the activity of neuro-regulatory systems with lasting behavioural consequences (Anda et al, 2006; Heim & Nemeroff, 2001; Teicher et al, 2003, 2014; Marusak, et al, 2015) EDT is known to affect brain development, including synaptic overproduction and pruning, myelination, and neurogenesis during sensitive and specific periods (Teicher et al, 2003). Function and structural neurobiological consequences of early stressful experiences have been shown (Teicher et al, 2003; Marusak et al, 2015). EDT appears to play a role in the aetiology of SAD. EDT may affect the development of the emotional systems of the brain, naimely the limbic circuitry, by greatly increasing its sensitivity and by allowing for the conditioning of automatic fear responses to neutral events by way of the amygdala.

### 2.2.1   SAD within the context of EDT

EDT is thought to reflect a unique neuroendocrine signature and a biological correlate of risk (Yehuda et al, 2010; Aas et al, 2012). Exposure to early stressors has been shown to lead to the persistent sensitization of the HPA axis and the central nervous system circuits, which are involved in human stress regulation. EDT is believed to affect the developing HPA axis by

sensitizing the brain to future stress reactions and making it hypersensitive to even minor stress reactions (Yehuda et al., 2010). This has been found to be true in studies that have assessed both abused children and adults that report EDT (Yehuda et al., 2010; Aas et al, 2012). A review of the literature on the effects of early stressors on brain development shows that early aversive experiences and the resultant stress response have profound effects on the developing brain resulting in impaired function of multiple brain structures and their functions (Anda et al., 2006; Teicher et al., 2003).

In earlier conceptualizations of the DSM-5, SAD was grouped with the fear circuitry disorders (Bögels & Stein, 2009). Little is actually known about individual and shared environmental factors that either promote or protect against the development of SAD and there little evidence for causative life events in its development (Bögels & Stein, 2009). EDT has been predominantly studied in posttraumatic stress disorder (PTSD) and obsessive compulsive disorder (OCD) (Heim & Nemeroff, 2001). Both of these disorders have been removed from the anxiety disorders category and being placed in their own separate disorder categories in the DSM-5 (APA, 2013). There is little known about the contribution of EDT to the presentation of SAD and there is a paucity of evidence for the aetopathophysiological mechanisms of EDT in SAD.

Magee (1999), in a retrospective study, examined the effects of life experiences on the onset of multiple types of phobias. The researchers found that patterns of chronic physical/verbal abuse during childhood had a significant impact on the onset of SAD. Bandelow et al. (2004) assessed a small sample of patients with SAD and EDT and found that EDT was reported significantly more frequently in individuals with SAD compared to healthy controls. Results from the Canadian Anxiety Disorders research program, which assessed 12 types of sexual abuse experiences occurring during childhood found that reports of sexual abuse were significantly more common in women with SAD than in healthy controls (Stein et al., 1996). Marteinsdottir, Svensson, Svedberg, Anderberg found similar results, and von Knorring (2007), where they investigated the relationship between life events and SAD during childhood and adulthood in a Swedish sample, as well as life events experienced in relation to the onset of SAD. Results showed that adversities, such as abuse during childhood, were significantly more frequent in SAD.

Kuo et al (2011) compared levels of childhood trauma in adults with generalized SAD versus

healthy controls and examined the relationship between specific types of childhood trauma and clinical symptomatology in SAD. Participants were 102 individuals with generalized SAD and 30 controls who completed measures of childhood trauma, social anxiety, trait anxiety, depression, and self-esteem. Compared to controls, individuals with SAD reported greater childhood emotional abuse and emotional neglect. Within the SAD group, childhood emotional abuse and neglect, but not sexual abuse, physical abuse, or physical neglect, were associated with the severity of social anxiety, trait anxiety, depression, and self-esteem (Kuo et al, 2011).

### 2.3 *Mechanisms underpinning SAD and EDT*

The effects of EDT on the pathophysiology of SAD, in the context of its epigenetic diatheses and associated neurobiological substrates, is complex.  These neurobiological mechanisms involve neurocognitive processes, specific brain circuitry, biochemical processes, structural morphemtric aberrations and specific genes.

### 2.3.1 *Neurocognitive functioning in SAD*

Meta-analyses indicate that information-processing difficulties characterize anxious individuals (Bar-Heim, et al 2007). It has been suggested that cognitive information processing difficulties, as represented by certain neuropsychological constructs, may underlie various emotional disorders, specifically SAD (Amir & Bomyea, 2010). In addition information-processing biases represented by neurocognitive difficulties may represent a causal role in the development and maintenance of SAD (Muckertz & Amir, 2014). Neurocognitive functioning may be represented by an individual's performance on various neuropsychological tests (Lezak et al, 2012). These performance measures may determine areas of cognitive weakness, fallout, or difficulty. Some domains of neurocognitive functioning may include functions such as memory, spatial processing, executive functioning, attention and verbal reasoning (Lezak et al, 2012).

In an American study, up to 49% of individuals with SAD left school prematurely (Ameringen, Mancini and Farvolden, 2003). It has been hypothesized that these difficulties were likely due to impaired cognition during stressful situations (Graver & White, 2007). Neurocognitive performance measures such as verbal attention, working memory and task shifting are significantly affected by anxiety (Paterniti, Dufouil, Bisserbe, and Alpeˊrovitch 1999). Anxiety disorders have also been strongly associated with hypothalamic- pituitary-

adrenal (HPA) axis dysregulation, which may underlie neurocognitive difficulties (Lee, Glass, McAtee, Wand, Bandeen-Roche, Bolla, et al., 2007). There has, however, been little attention payed to the neurocognition of anxiety disorders and SAD specifically (Graver & White, 2006; Sutterby & Bedwell, 2012). There are also very few studies examining neurocognitive difficulites with regards to anxiety and social anxiety in non-clinical samples and populations (Castaneda, Suvisaari, Marttunen, Pera, Saarni, Aalto-Seta, Lonnqvist & Tuulio-Henriksson, 2011).

Although there are a limited number of studies focusing on the neurocognitive functioning of SAD (O'Toole & Pederson, 2011), most of these have focused predominantly on attention and memory processing (Amir & Bomyea, 2010; Muckertz & Amir, 2014). These specific areas of deficit are also supported by numerous neuroimaging studies that have implicated the temporal lobes, hippocampi and amygdala (Airaksinon, 2004; Freitas-Ferrari et al, 2010). Memory processes appear to play a substantial and important role in the maintenance of social fears and SAD specifically (Heinrichs & Hoffman, 2004). In early investigations of the neuropsychology of SAD, it was also assumed that individuals with SAD process information and specifically encode information in a biased way (Beck & Clark, 1997).

The effect of state factors on neurocognitive performance in SAD has not been thoroughly investigated and the overall neurocognitive profile remains poorly understood (O'Toole et al, 2014). O'Toole et al (2014) examined the effects of state anxiety and state emotion suppression on neurocognitive performance in 42 participants with SAD and 42 gender and education matched controls, and found participants with SAD performed worse than controls on processing speed, visuospatial construction, visuospatial memory, verbal learning and word fluency, of which only the decreased visuospatial construction performance was considered clinically significant. State anxiety was not associated with neurocognitive performance at baseline, whereas state emotion suppression predicted decreased visuospatial memory in controls and decreased verbal learning in the SAD group (O'Toole et al, 2014). Both groups performed better on working memory, processing speed and spatial anticipation, and worse on verbal learning and memory following an anxiety manipulation task. An increase in state anxiety was associated with a decrease in verbal learning in both groups. It can therefore be stated that participants with SAD showed clinically significant difficulties with visuospatial construction and may experience verbal learning difficulties when suppressing emotions and experiencing an increase in anxiety (O'toole et al, 2014).

During stressful events individuals who have been diagnosed with generalized SAD perform more poorly on neurocognitive working memory tasks compared with individuals who do not have generalized social anxiety disorder (Graver & White, 2007) A recent study of memory examined the encoding processes that relate to another personally relevant subsequent memory task (Vassilopoulos, 2012). Vassilopoulos (2012) asked children who were high and low on social anxiety to rate a series of positive and negative words in terms of how well the words described themselves. High socially anxious children were able to recall fewer positive social words compared to low socially anxious children (Vassilopoulos, 2012). This may be indicative of difficulty in memory encoding positive information prior to recall or that enhanced recall for negative information may be related to social anxiety (Amir, 2014).

Graver and White (2006) found in their investigation of neurocognitive differences between SAD and controls that spatial span scores were reduced for the SAD group during stress; was improved in the healthy controls; and demonstrated no change in individuals with comorbid depression and SAD. Trail making test times showed an interaction effect, with completion time improving significantly less for the SAD group than the other groups and executive function also showing a significant decline in the SAD group compared with the other two groups (Graver & White, 2006).

Airaksinon, Larsson and Forsell (2004) examined various neurocognitive difficulties in a wide range of anxiety disorders: panic disorder with and without agoraphobia, OCD, SAD and specific phobias. The authors looked specifically at episodic memory, verbal fluency, psychomotor speed, and executive functioning. They found that in the total anxiety disorders sample there were deficits both in executive functioning and episodic memory. However there appeared to be only episodic memory difficulties within the SAD group when this group was separated out from the total anxiety disorders group (Airaksinon et al, 2004).

Castaneda, et al. (2011) compared neurocognitive test findings of individuals diagnosed with an anxiety disorder and compared them to control subjects without an anxiety disorder. They examined verbal and visual short-term memory, verbal long-term memory, attention, psychomotor processing speed, and executive functioning. The authors found that there was no significant difference in neurocognitive test scores between the two groups. However when they excluded participants who were in remission, they did find that those with an

anxiety disorder scored lower on visual working memory tests. They also found that the current use of psychotropic medication and low current psychosocial functioning was associated with deficits in executive functioning, psychomotor processing speed, and visual short-term memory (Castaneda et al., 2011).

However some investigations have yielded evidence that may be contrary to what has been suggested with regards to a bias in information coding. It may not be that an activated anxiety schema results in better encoding of threat-related information (Beck, Emery, & Greenberg, 1985), but rather that there may be less elaborative encoding of threat-information as there may not be a mood-congruent explicit memory bias (Russo, Fox, Bellinger, and Nguyen-Van-Tan 2001; Williams, Watts, MacLeod, & Mathews, 1997).  Also Asmundson, Stein and Larson (1995) have found significant memory recall deficits for verbal information.

Interestingly with regards to memory processing and encoding, Heinrichs and Hofmann (2004) found that memory encoding for threat information in 'high' socially anxious individuals was poorer than in individuals who were 'low' on social anxiety. In their study they examined release from a proactive interference technique to explore memory encoding in SAD. The assumed hypothesis was that memory encoding for social threat would be higher in individuals with high social anxiety rather in those who were low on social anxiety (Heinrichs & Hofmann, 2004).

There are some contrary neurocognitive findings that do not support the deficits found in neurocognitive studies of SAD. A recent study examined a series of 9 neurocognitive assessment instruments in 25 individuals with generalized SAD (Sutterby & Bedwell, 2012) and found no significant neurocognitive performance difficulites. There results indicated that there were no underlying neurocognitive difficulites compared to healthy controls. Their conclusion was that neurocognitive deficits are unlikely to account for the information processing biases in SAD (Sutterby & Bedwell, 2012). Coles and Heimberg (2002) found no significant memory biases, specifically for threat relevant information, in SAD compared with other anxiety disorders (panic disorder, posttraumatic stress disorder and obsessive compulsive disorder). Other studies have found no significant impairments in memory processing in individuals with SAD (Lundh & Ost, 1993; Rapee, McCallum, Melville Ravenscroft & Rodney, 1994). In these studies various neurocognitive tests across the various domains of memory did not show any significant difference between individuals

diagnosed with SAD and healthy controls.

Attentional biases have been reported in individuals with SAD (Roth, 2004). This has been shown in individuals with SAD who demonstrate more attention to threat-relevant words, in various Stroop studies (Lundh & Ost, 1996; Hope, Rapee, Heimberg, & Dombeck, 1990; Maidenberg, Chen, Craske, Bohn, & Bystritsky, 1996; Mattia, Heimberg, & Hope, 1993). Individuals with SAD are slower at colour naming socially relevant threat sensitive words than socially relevant neutral words than controls (Amir, Freshman & Foa, 2002; Spector Pecknold & Libman, 2003).

Individuals with SAD are also less likely to attend to facial expressions than controls, indicating anxiety and stress mediated attentional bias (Roth, 2004; Horley, Williams, Gonsalvez & Gordon, 2003). Individuals with SAD have also been found to have more impaired verbal attention compared to controls. However spatial attention was mostly intact (Asmundson, Stein, Larsen, & Walker, 1994). In another study by Cohen et al. (1996) individuals with SAD where found to have lower spatial working memory and task shifting performance than controls.

There is a significant relationship between EDT and later cognitive functioning (Majer et al., 2010). Stressful and aversive experiences such as EDT could ultimately lead to difficulties in cognitive functioning in individuals with SAD. It was previously mentioned that SAD populations report significantly higher rates of EDT compared to healthy controls. Bremner et al. (1995) assessed the short term memory capacity of 20 survivors of severe childhood sexual and physical abuse and 21 controls and found significantly lower scores on the logical memory scales of the Wechsler Memory Scale for immediate and delayed recall in the EDT group. Deficits in verbal memory were associated with the severity of abuse. The authors concluded that trauma has a long-lasting negative effect on verbal memory (Bremner et al., 1995).

In addition Majer et al examined 47 healthy adults, who formed part of a larger study conducted in Unites States using the Cambridge Neuropsychological Test Automated Battery (CANTAB) in combination with the Wide-Range-Achievement-Test (WRAT-3) (Majer et al., 2010), and the Childhood Trauma Questionnaire (CTQ). The study was conducted to assess cognitive functioning and with regards to type and severity of EDT. They found that

physical neglect and emotional abuse was associated with deficits in memory functioning (Majer et al., 2010).

### 2.3.2 *Brain circuitry in SAD*

There are a number of brain circuits involved in the pathophysiology of SAD. These brain circuits are interconnected and are involved to various degrees in SAD. The cortico-limbic circuitry has been the most substantially studied in SAD and its dysregulation is key in SAD pathophysiology (Warwick et al., 2008; de Vente, Majdandzic & Bogels, 2014; Talati et al, 2013, 2015). The cortico-limbic region comprises interconnected structures involved with emotion, motivation and memory (Detweiler, Comer & Albano, 2014). In addition, frontal lobe function has also been found to play a significant role in modulating the increased limbic activity associated with SAD (Warwick, et al., 2008; Freitas-Ferrari et al, 2010). One particular anatomical structure in the limbic system, the amygdala (see Figure 2), plays a significant role in fear learning and in social fears in particular and is principally related to the pathophysiology of SAD (Birbaumer et al., 1998; Etkin & Wager, 2007; Rosen & Schulkin, 1998; Shin & Liberzon, 2010; Detweiler, Comer & Albano, 2014; de Vente, Majdandzic & Bogels, 2014).
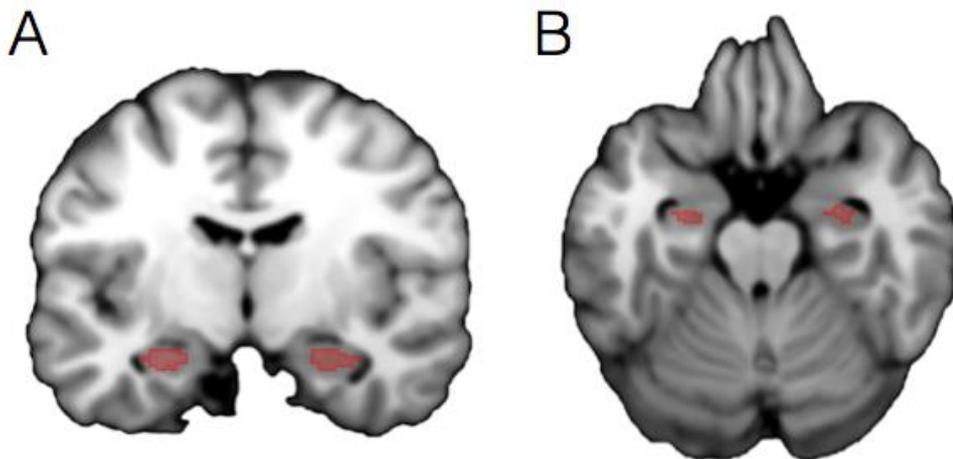


**Figure 2** is a coronal (**A**) and axial (**B**) section taken from Mango ROI imaging software program showing the right and left amygdala and their position in an MRI T1 weighted image.

Anatomically the amygdalae (plural, [referred in this paper in the singular – amygdala]), also known as the *corpus amygdaloideum*, are a pair of small almond shaped structures, located medial to the hypothalamus and adjacent to the hippocampus and within the anterior portion of the temporal lobes (Cozolino, 2002; Lezak et al., 2012) (see Figure 2). Most research on

the histology and anatomy of the amygdala has been conducted on non-human primates and rodents. However, the variations between human and other primate amygdala appear to be minor (Whalen & Phelps, 2010). The amygdala is also considered to be one of the central limbic structures associated with fear learning (Whalen & Phelps, 2010; LeDoux, 2000; Talati et al, 2013, 2015). It consists of a number of nuclei with differing input and output pathways. Through these various inputs and outputs, the amygdala has connections with the cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, and brainstem nuclei (Lezak et al., 2012; Whalen & Phelps, 2010). Importantly, the basolateral portion of the amygdala has evolved with the expansion of the cerebral cortex. Its integration with the cerebral cortex includes its processing of sensory information, especially visual information (Cozolino, 2002; Whalen and Phelps, 2010). The amygdala is primarily involved in emotional learning and approach-avoidance strategies (LeDoux, 2000). It functions by incorporating fear experiences into its repertoire of responses. The central nucleus of the amygdala is the primary center where sensory experiences become associated with fear and is therefore the principle site of fear processing and learning (Cozolino, 2006). Projections from the amygdala to the lateral hypothalamus form the basis for the activation of the sympathetic fear response; this generally involves increases in heart rate and blood pressure (Cozolino, 2006). The amygdala has been considered to be one of the most important components in the evolutionary processes of fear learning (Whalen & Phelps, 2010).

Throughout evolution, neural systems evolved to support an increase in survival (Cozolino, 2006).  The amygdala expanded during the evolutionary development of the brain to incorporate more complex cognitive, emotional and sensory inputs (Whalen & Phelps, 2010). The amygdala is predominantly involved in the safety responses pertaining to flight and fight. The flight and fight networks incorporate the various affective, memory and social networks. The amygdala is an important component of emotion, motivation and memory processing and is intrinsically important in the processing of both social cues and emotional expression in interpersonal functioning. It is therefore principally involved in the sympathetic nervous system response to anxiety, especially with regards to social anxiety (Birbaumer et al., 1998; Etkin & Wager, 2007; Rosen & Schulkin, 1998; Shin & Liberzon, 2010, Detweiler, Comer & Albano, 2014). Classical conditioning models of fear learning have been used to explain some of the processes in which the amygdala associates specific sensory stimuli to fear elicitation or activation of central nervous system arousal pathways (Whalen & Phelps, 2010), especially in situations concerning social fear (Stein & Stein, 2008).

It is important to note that damage to the amygdala typically leads to profound deficits in social functioning and emotional processing, such as problem solving in interpersonal situations and the ability to discern accurate emotional face recognition and body language (Ammerlaan, Hendriks, Colon, & Kessels, 2008; Cristinzio, N'Diaye, Seeck, Vuilleumier, & Sander, 2010; Scott et al., 1997; Shaw et al., 2004; see also Corden, Critchley, Skuse, & Dolan, 2006, Detweiler, Comer & Albano, 2014). When individuals with SAD encounter situations that they perceive as socially threatening, there is typically an increase or over responsiveness in amygdala activation (Phan et al., 2006; Adolphs, 2003). As perceived social threat increases, the amygdala responds to facial cues with an increase in activity (Morris et al., 1996, Detweiler, Comer & Albano, 2014). There is a positive correlation between the severity of social anxiety and the degree of amygdala attenuation when individuals are presented with social-threat cues. This has been demonstrated both in individuals diagnosed with SAD (Phan, Fitzgerald, Nathan, & Tancer, 2006) and in individuals without a SAD diagnosis, but who have high behavioral inhibition (BI), a risk factor for a possible diagnosis of SAD (Pérez-Edgar et al., 2007).

The neural systems involved in social fear processing and interpretation of social threat may be affected by earlier life events, such as EDT. EDT may affect the neuroplascticity and functioning of the amygdala (Grant et al, 2011; Corbo et al, 2014). The underlying pathophysiology of SAD within the context of EDT may not only incorporate structural alterations of the amygdala. There may be specific functional changes in the amygdala significant of EDT in SAD. When examining the interplay between these fear conditioned processes and brain morphometry of the amygdala, researchers have predominantly used neuroimaging modalities in examining these brain circuits (Freitas-Ferrari et al., 2010).

### 2.3.3 *Neuroimaging in SAD*

Neuroimaging has contributed to a better understanding of the neurobiology of SAD (Freitas-Ferarri et al, 2010). The few neuroimaging studies performed in SAD, range from structural magnetic resonance imaging (sMRI) studies, tractography, fMRI, ^{1}H-MRS, SPECT, PET and relaxometry (Fretias-Ferrari, Hallak, Trzesniak, Filho, Machado-de-Sousa, Chagas, Nardi and Crippa (2010). In a review of the literature on neuroimaging of SAD, Freitas-Ferrari et al. (2010) examined 48 studies of SAD in the context of various cognitive tasks and

provocations, including facial expressions of emotion, symptoms provocation paradigms, and disorder-related abnormalities of serotonin or dopamine neurotransmission. The commonest modality appears to be facial emotion tasks in fMRI followed by PET and then SPECT studies. It appears that fMRI is the most widely used neuroimaging methodology in SAD, because it has higher image resolution and enables researchers to distinguish more easily between different brain tissues and regions, it also importantly allows for multiple measurements in a single imaging session (Freitas-Ferrari et al., 2010). fMRI also does not have the limitations of PET and SPECT studies. Both PET and SPECT use gamma rays produced by radiolabeled tracers that reach the brain after intravenous injection or inhalation (Freitas-Ferrari et al., 2010). Structural magnetic resonance imaging (sMRI) is one of many brain-imaging techniques used to visualize the internal structures of the brain (Deichmann et al, 2000).

sMRI enables measurement using the detection of spinning protons ($^1$H), which are the nuclei of hydrogen atoms in the molecules that make up the various tissues of the human brain (Deichmann et al, 2000). The detection of $^1$H is based on a physical property called 'spin'. Spin can be described as the aspects of momentum and magnetization properties of $^1$H (Deichmann et al, 2000). A number of statistical techniques may be employed in order to examine the variation between brain structures in individuals with specific anxiety disorders compared with healthy controls (Hurley, Fisher, Taber, 2008). There have been few structural imaging studies of SAD (Pannekoek, et al, 2013; Freitas-Ferrari et al. 2010), those that have been conducted have used varying approaches and have reported inconsistent results. Early sMRI studies did not show brain volume differences between SAD participants and controls (Potts, Davidson, Krishnan & Doraiswamy 1994; Phan & Klumpp, 2014). However more recent studies have indicated reduced amygdala and hippocampal volumes in adults (Irle et al, 2010) and in adolescents with SAD (Mueller et al., 2013). For example, some studies have demonstrated no abnormalities and other studies, decreases in the volume of the amygdala, posterior right temporal gyrus and hippocampus (Irle et al., 2010; Liao et al., 2011; Potts et al., 1994, Pannekoek, et al., 2013).

A number of grey matter (GM) abnormalities have been identified in SAD (Talati, Pantazatos, Schneier, Weissman & Hirsch, 2013). Compared with healthy controls, abnormalities in SAD range from increased GM in the left parahippocampal, middle occipital, bilateral supramarginal and angular cortices and the left cerebellum; and lower GM

in the bilateral temporal poles and left lateral orbitofrontal cortex (Talati et al, 2013). Cerebellar, parahippocampal, and temporal pole differences have also been observed in SAD subgroups compared with healthy controls (Talati et al., 2013).

Structural brain deficits in relation to EDT have also been found. A study assessing childhood aversive events, ACC, hippocampus, amygdala and caudate nucleus volumes in 265 healthy Australian men and women with regards to aversive childhood events (Cohen et al., 1996) found that individuals who reported more than two aversive events had significantly smaller ACC and caudate nucleus volumes compared to individuals with less aversive experiences. These effects were not found for hippocampal or amygdala volumes (Cohen et al., 1996).

It is important to note that there are some studies, which have not documented changes in the amygdala or the hippocampus in individuals with SAD (Potts et al., 1994; Syal et al, 2012; Phan & Klumpp, 2014). In a sMRI study by Potts et al. (1994), they examined caudate, putamen, thalamus and whole brain ROI analyses in 22 SAD participants with age and sex matched controls and found no significant differences in volumes between SAD participants and controls. An important component recently investigated, which has shown smaller volume differences with increased symptom severity, is the ACC in individuals with SAD compared with healthy controls (Frick et al, 2013). In addition in an fMRI study less ACC responsiveness has been shown in SAD compared with controls when asked to look away from distracting emotional faces (Klumpp, Post, Angstadt, Fitzgerald & Phan, 2013). Functional neuroimaging studies may provide insights about the processing of structural abnormalities found in SAD.

Functional neuroimaging broadly encompasses a number of modalities that examine brain activity during a specific period of activation or stimulus presentation. Most studies allow for the in-vivo investigation of neural activation, in order to understand the specifics of cognitive functioning and disease presentation in the brain. The modalities include positron emission tomography (PET), magnetic encephalography (MEG), electroencephalography (EEG), single photon computed tomography (SPECT), and functional magnetic resonance imaging (fMRI). Currently the field of fMRI is gathering much attention, including the anxiety disorders field and will be covered in this review of the SAD literature. Most fMRI studies in anxiety have focused on subcortical brain activations or the relationship between subcortical brain regions and specific cortical regions (such as frontal-striatal function).

Although there have been many functional neuroimaging studies in anxiety disorders, few have specifically investigated SAD. Neuroimaging studies of SAD include PET, SPECT and fMRI (Amir et al., 2005; Furmark et al., 2002; Phan et al., 2006; Stein et al., 2002; Tillfors et al., 2001). These functional neuroimaging techniques have revealed that the amygdala and the insular cortex and the hippocampus may play a substantial role in the pathophysiology of SAD (Nakaoa, Sanematsua, Yoshiurab, Togaob, Murayamaa, Tomitac, Masudac, & Kanbaa, 2011). Etkin and Wager (2007) in a meta-analysis of functional neuroimaging studies revealed that the typical fear circuits were overactive in SAD.

Early fMRI studies have shown enhanced amygdala activation in response to neutral (non-aversive) faces in individuals with SAD (Syal & Stein, 2014; Birbaumer et al., 1998). Also early work has found memory bias for critical facial expressions (Lundh & Ost, 1996) and subsequent functional imaging studies found activation for angry and contemptuous faces in individuals with SAD relative to controls (Stein, Golding, Sareen, Zorilla & Brown, 2002). Exaggerated amygdala response to negative facial affect has been observed in numerous fMRI studies in SAD (Phan, Fitzegerald & Nathan, 2006; Straube Kolassa, Glauer, Mentzel & Miltner, 2004), including a study that examined the processing of schematic faces (ie. Line drawings) (Evans, Wright & Wedig, 2008). Moreover, the magnitude of amygdala activation to threatening faces is generally, positively correlated with the severity of SAD symptoms (Phan et al, 2006) and the successful treatment of SAD is associated with a decreased blood flow to the amygdala during performance of a public speaking tasks (Furmark et al, 2002).

In a review by Freitas-Ferrari et al. (2010), the authors found the amygdala and its connections (in the emotional and fear circuitry) to play a key role in SAD (Freitas-Ferrari et al., 2010). The insula (Shah et al., 2009), ACC (Amir et al., 2005; Blair et al., 2011) and ventromedial prefrontal cortex have also been reported to be involved in SAD (Damsa et al., 2009; Etkin and Wager, 2007; Freitas-Ferrari et al., 2010, Pannekoek, et al., 2013). For example less ACC responsiveness has been shown in SAD compared with controls when asked to look away from distracting emotional faces (Klumpp, Post, Angstadt, Fitzgerald & Phan, 2013).

Methodological developments such as functional connectivity and more standardized

structural analyses of grey and white matter have been developed to further investigate fear circuitry in SAD (Bruhl et al, 2014). The amygdala has specifically been a focus on investigation, as it is considered a key structure in fear learning and in the pathophysiology of SAD (Frietas-Ferrari et al, 2010). Functional connectivity studies are designed to examine the relationship between various neural structures in the brain, either during a task or during states of rest. Research by Pannekoek et al. (2013) demonstrated differences in functional connectivity of the amygdala and in areas specifically involved in self-awareness in drug-naive SAD patients without comorbidity compared with controls. Some of their specific findings indicated that individuals with SAD demonstrated increased negative right amygdala connectivity with the left middle temporal gyrus. They also had an increase in connectivity in the left supramarginal gyrus and left lateral occipital cortex. With regards to the salience network, individuals with SAD  also showed increased positive bilateral dorsal ACC connectivity with the left precuneus and left lateral occipital cortex compared with controls (Pannekoek, et al, 2013).

Alterations have been found within the amygdala functional connectivity network in SAD (Hahn, et al, 2011). Amygdala hyperactivation has been associated with dysfunction of fronto-amygdala network inhibition and attenuation of emotional processing. This has been observed to have a specific impact on the modulatory influence of the ACC within the fronto-amygdala network, on threat perception and processing (Hahn, 2011). For example in a study by Hahn et al (2011), of 10 SAD participants matched with 27 controls, SAD participants exhibited amygdala hyperactivation during an emotion task and decreased functional coupling of the left amygdala with the medial orbitofrontal cortex and the posterior cingulate cortex/precuneus. The strength of the functional connectivity showed a negative association with the severity of anxiety experienced. In addition, further exploratory analysis revealed reduced functional connectivity and a marked functional separation between the medial orbitofrontal and ACC in the SAD group (Hahn et al, 2011).

The limbic system is not the only neural system implicated in the pathophysiology of SAD, for example observations of alterations in pre-frontal regions and reduced activity in striatal and parietal areas show that much remains to be investigated (Freitas-Ferrari et al, 2010). The medial prefrontal cortex provides additional support for a corticolimbic model of SAD pathophysiology, and is a promising area to investigation (Freitas-Ferrari et al, 2010). In

addition, pre/post treatment studies have found decreased perfusion in the same areas after either pharmacological or psychological treatment (Freitas-Ferrari et al 2010).

Although methodological differences abound across neuroimaging studies, findings have often been consistent, reinforcing evidence for specific neural circuitry in SAD, formed by limbic and cortical areas (Freitas-Ferarri et al, 2010). Bruhl et al (2014) have provided a comprehensive update and a meta-analysis of neuroimaging studies in SAD and present a new model of the neurobiology of SAD. Meta-analytic findings confirm hyperactivation of the fear circuit of the amygdala, as well as activation in the insula, ACC and prefrontal cortex in SAD. In addition, there is hyperactivation of medial parietal and occipital regions (posterior cingulate, precuneus, cuneus) in SAD and reduced connectivity between parietal and limbic and executive network regions (Bruhl et al, 2014).

SAD and panic disorder have been associated with aberrant amygdala responses to threat-related stimuli. In both disorders, there is considerable association with amygdala function and its connectivity with medial prefrontal cortex (mPFC) during emotional face perception (Demenescu et al, 2014). Demenescu et al  (2014) examined 14 participants with panic disorder, 17 particpants with SAD, 8 participants with comorbid panic disorder and SAD, and 16 controls, blood oxygen level dependent responses of perceiving emotional facial expressions. They found that panic disorder, but not SAD, was associated with amygdala and lingual gyrus hypoactivation during perception of angry, fearful, happy and neutral faces, compared to healthy participants. No significant effect of panic disorder and SAD diagnosis was found on amygdala mPFC connectivity. A positive correlation of anxiety symptom severity was found on amygdala-dorsal anterior cingulate and dorsal mPFC connectivity during perception of fearful faces. Symptom severity, but not the presence of panic disorder and an SAD diagnosis per se, explained most of the abnormalities in amygdala mPFC connectivity during perception of fearful faces (Demenescu et al, 2014).

The changing pattern in functional network hubs in SAD remains incompletely understood. Network hubs, are characterized by a large number of connections between specific neural circuits in the brain and play an important role in specific patterns of functional activation (which characterize SAD). Previous functional neuroimaging studies have demonstrated aberrant functional connectivity in SAD characterized by specific network hubs (Liu et al, 2014).   Liu et al (2014) conducted a study of 20 SAD participants and 20 matched controls

examining resting-state fMRI data of functional networks by measuring the temporal correlations of each pair of brain voxels. Hubs were identified using a graph theory method. Compared with healthy controls, SAD participants showed significantly decreased functional connectivity strength in the bilateral precuneus and significantly increased functional connectivity strength in the right fusiform gyrus. Furthermore, a significantly negative correlation was observed between the functional connectivity strength value in the precuneus and illness duration.   The present study demonstrated for the first time that disrupted cortical network hubs exist in individuals with SAD during resting state (Liu et al, 2014).

There is a paucity of research on local synchronization of spontaneous functional magnetic resonance imaging (fMRI) blood oxygen level-dependent (BOLD) signals that occur in SAD during the resting state. Qiu investigated altered neural activity in resting state using a regional homogeneity (ReHo) analysis on 20 SAD and 20 healthy controls. Compared with healthy controls, SAD participants exhibited decreased coherence (ReHo) in the bilateral angular gyrus and the left medial prefrontal cortex within the default mode network (DMN), suggesting functional impairment of the perception of socially relevant emotional state and self-related mental representations; and also in the right dorsolateral prefrontal cortex and right inferior parietal gyrus within the central-executive network (CEN), reflecting a deficit of cognitive control of social anxiety. Significantly increased coherence was found in the left middle occipital gyrus, which would be consistent with their hypervigilance and hyperprosexia to social communication even in the resting state.

Another example of neural network differences in SAD, besides in the amygdala and limbic system, may be demonstrated in the The Default Mode Network (DMN) (Qiu et al, 2011; 2014). The DMN is a constellation of brain areas that decrease their activity during a wide number of different goal-oriented tasks as compared to passive "rest" tasks (Gentili et al, 2009). DMN can be modulated by different factors such as emotional states, cognitive load of the task and psychopathology, including levels of anxiety. Moreover, DMN seems to play a pivotal role in social cognition and is therefore important in understanding SAD. The ability to predict another person's behavior taking his or her perspective modulates the activity of the DMN. Recent data from autistic patients support a role of DMN in social cognition as well. There have been very few studies that have assessed DMN in SAD. Gentili (et al, 2009) showed a lower deactivation in the precuneus and posterior cingulate regions (PCun/PCC) during task conditions in SAD participants compared to controls. These regions are part of

the so-called "*Theory of Mind*" circuit and in particular they are involved in the evaluation of one's own emotional state (Gintili, 2009). Because of the role of the PCun/PCC in self-state perception and attribution and, more in general, the role of the DMN in social cognition, we suggest that its impairment in the DMN network in SAD might be relevant in the development of the feeling of wariness of others' judgment and may be related to the so-called self-focused attention (Gentili et al, 2009).

2.3.4 *Neurometabolism in SAD*

Most studies of neurotransmitters and many that have examined neurometabolites have been in vitro. However, recent advances in neuroimaging modalities that are non-invasive have allowed researchers to examine neurotransmitters and neurometabolites in vivo (de Graaf, 2007). Understanding neurometabolism may provide a window into the pathophysiological processes of the cellular machinery involved in psychopathology, in specific target anatomical structures. ^{1}H-MRS measurements of the amygdala could provide insight into the neuronal and glial differences that compose the gross volumetric and functional changes in normal and abnormal developmental pathways; greatly enhancing understanding of this major target of psychiatric and behavioral therapy (Nacewicz, et al. 2012).  Neuroimaging forms an important methodological tool, together with clinical and neurocognitive investigations in understanding the neurobiological mechanisms of SAD. This may be especially important in understanding the neurobiological mechanisms of SAD within the context of EDT.

^{1}H-MRS is an analytical technique that can be used to complement MRI in the molecular characterization of brain tissue in vivo. Both ^{1}H-MRS and MRI techniques use signals from ^{1}H, where MRI uses the information to create 2-dimensional images of the brain and ^{1}H-MRS uses ^{1}H signals to determine the relative concentrations of target brain neurometabolites, in a regionally specific manner. In addition ^{1}H-MRS permits the in vivo determination of neuronal density and functional integrity of specific target anatomical structures (Mathew et al, 2003). The neurometabolites frequently studied in anxiety disorders and their pathophysiologies consist of: inositol (Ins), N-acetylaspartate (NAA), Glutamate (Glu), Glutamine (Gln), Creatine (Cr) and Phosphocreatine (PCr) (de Graaf, 2007). A small body of research has focused on a number of neurotransmitters and neurometabolites in SAD (Bandelow & Stein, 2005). Some important developments in understanding the neurobiology of SAD have been made in the neuroimaging field, specifically in ^{1}H-MRS (Phan et al.,

2005; Howells et al, 2015). However there are currently few ^{1}H-MRS studies of SAD (Howells et al, 2015).

Of the neurometabolites and neurotransmitters that have been studied, there is substantial evidence to support the involvement of Glu in anxiety disorders (Harvey & Shahid, 2012; Bermudo-Soriano et al, 2012) and of the neurometabolites studied in vivo, Glu has been of particular interest in SAD (Phan et al., 2005). Bermudo-Soriano et al. (2012), in their comprehensive review, found considerable clinical evidence for the role of glutamate in anxiety disorders and found that there was an elevation in Glu in individuals with anxiety and social fears (Bermudo-Soriano et al., 2012).  Glu is a nonessential amino acid with multiple roles in vivo (de Graaf, 2007; Erecinska & Silver, 1990). It plays a very important role in neurotransmission of the inhibitory neurotransmitter GABA, as a precursor of GABA (de Graaf, 2007). It also plays an important role in the synthesis of other neurometabolites such as glutathione (de Graaf, 2007) and other larger neuropeptides and proteins used in neurotransmission (Erecinska & Silver 1990).

Glu is one of the most abundant neurotransmitters of excitatory synaptic activity (Hammond, 2008). The main activity of the glutamate system is to activate G-proteins upon glutamate binding (Hammond, 2008). With regards to the anxiety disorders, glutamate plays a specific role in modulating affective arousal. Metabatropic Glu is involved in synaptic excitability and synaptic transmission (Hammond, 2008). Glu is a major excitatory neurotransmitter, of the mammalian brain (de Graaf, 2007; Erecinska & Silver, 1990), responsible for approximately 40% of all neurotransmission (Coyle et al., 2002 cited in Kent, Mathew & Gorman 2002). There are significant differences in Glu concentrations between grey and white matter, with higher concentrations occurring in grey matter regions. Glu occurs in larger concentrations in glutamatergic neurons and smaller concentrations in GABAergic neurons and astroglia (de Graaf, 2007). Glutamine (Gln) is a neurometabolite that plays an intermediary role in the neurometabolism of Glu in the astroglia of neurons (de Graaf, 2007). Gln is synthesized from Glu by Gln synthetase in the astroglia and it is broken down to Glu by phosphate-activated glutaminase in neurons (de Graaf, 2007).

A growing literature on the neurobiology of SAD has suggested that the reward/avoidance basal ganglia circuitry in general and the glutamatergic system in particular may play a role in its pathophysiology (Howells et al, 2015). Only two ^{1}H-MRS studies have measured

concentrations of Glu in SAD participants, and these found increased Glu relative to Cr in the ACC (Phan et al., 2005) as well as increased Glu/Cr for the whole brain (Pollack et al., 2008). There is currently good evidence that Glu is involved in fear conditioning and in the neurobiological response to aversive stimuli (Harvey and Shahid, 2012). There is also considerable evidence from animal studies of the strong connection between stress and brain Glu levels in changes in neuroplasticity (Harvey and Shahid, 2012, Spedding et al., 2003). This evidence also supports the role of Glu in the pathogenesis of anxiety disorders (Harvey and Shahid, 2012; Cortese and Phan, 2005; Krystal et al., 2010).

Phan et al. (2005) found a correlation between the magnitude of Glu in the ACC and the severity of social anxiety symptoms. Glu has also been observed to be higher in the prigenual ACC than controls, however no difference was found in Glu in the occipital cortex (Phan et al, 2005). Also, the level of Glu has been demonstrated to be decreased by the addition of anxiolytic treatment (Pollack et al., 2008). Furthermore, a number of studies have demonstrated the effects of various psychoactive drugs that target glutamate in anxiety; such as memantine in generalized anxiety disorder (GAD) (Feusner et al., 2009), liluzole (Mathew et al., 2008), metabotropic glutamate receptor (2/3) agonists (Dunayevich et al., 2008) and pregabalin (Baldwin and Ajel, 2007). There is even some evidence for the efficacy of glutamatergic agents- D-serine and D-cycloserine- as an adjunct to cognitive behavioral therapy (CBT) in posttraumatic stress disorder (PTSD) (Heresco-Levy et al., 2002, 2009; Harvey and Shahid, 2012).

NAA, another neurmatebolite, is exclusively localized in the nervous system (de Graaf, 2007). Its concentration can vary across different areas of the brain and may change developmentally. NAA appears to be involved in osmoregulation of the neurotransmitter *N*-acetylaspartylglutamate (NAAG) (Blakely, 1988; de Graaf, 2007) and is a marker of neuronal density and functional integrity (Barker, 2001). Some studies have shown area specific decreases of NAA in the brain in a variety of neuropsychiatric and cognitive disorders (Clark, 1998; Ferguson et al., 2002; Friedman et al., 1998; Jung et al., 1999; Ross and Sachdev, 2004; Tsai and Coyle, 1995 Ariyannur, 2008); these reductions may constitute compromised neuronal integrity or fitness (Barker, 2001; Bertolino et al., 1997, 2002).

^{1}H-MRS studies have also indicated increased absolute NAA and/or NAA/creatine concentrations in the frontal cortex of anxious participants without psychopathology

(Grachev & Apkarian, 2000) and in the ACC of participants with SAD (Phan et al, 2005). There is also evidence of a significant positive correlation between severity of SAD symptoms and NAA/creatine ratios in cortical grey matter (Tupler et al, 1997). However a similar study found the opposite ratio in NAA in both cortical and sub-cortical regions (Davidson et al, 1993). Ins has also been observed to be significantly higher in subcortical grey matter and white matter in SAD (Tupler et al, 1997). Thus far two ^{1}H-MRS studies in SAD have determined correlations between neurometabolite concentrations with measures of SAD. The one study found Glu/Cr concentration in the right dorsolateral prefrontal cortex to be positively associated with increased illness severity (Yue et al., 2012). The second study found decreased Cr concentrations in the ACC and was associated with intensity in SAD fear/anxiety (Phan et al., 2005).

There is recent evidence in a ^{1}H-MRS study that the reward/avoidance basal ganglia circuitry, as well as the glutamatergic system, play a role in mediating SAD symptoms (Howells et al, 2015). Howells et al (2015) investigated ^{1}H-MRS neurometabolite concentrations in cortical, striatal, and thalamic circuitry as well as their associations with clinical measures of SAD, in 18 SAD participants and 19 age- and sex- matched controls. They focused predominantly on the ACC using single voxel spectroscopy, reporting on NAA with N-acetyl-aspartyl-glutamate (NAA + NAAG), glycerophosphocholine with phosphocholine (GPC + PCh), Ins, Glu, and Glx (Glu + Gln), and in the caudate, putamen and thalami bilaterally, using two dimensional chemical shift imaging (reporting relative NAA + NAAG and GPC + PCh). The clinical measures of SAD included symptom severity, blushing propensity, and gaze anxiety/avoidance. They found, first, decreased relative glutamate concentration in the ACC of SAD and changes in Ins with measures of social anxiety. Second, NAA metabolite concentrations were increased in the thalamus of SAD, and choline metabolite concentrations were related to measures of social anxiety. Lastly, choline metabolite concentration in the caudate and putamen showed changes in relation to measures of SAD symptom severity (Howells et al, 2015).

Serotonin is a neurotransmitter that has been a focus of attention in SAD, due to the efficacy of selective serotonin re-uptake inhibitors in the treatment of SAD (Ameringen & Mancini, 2004). A significant reduction in 5-HT1A binding in the amygdala has also been observed in anxiety and in social anxiety (Cited in Lanzenberger, Mitterhauser, Spindelegger, Wadsak, Klein, Mien, Holik, Attarbaschi, Mossaheb, Sacher, Geiss-Granadia, Kletter, Kasper &

Tauscher, 2006). Lanzenberger et al. (2006) also found significantly lower 5-HT1A receptor binding potential in male patients suffering from SAD than in an age-matched healthy control group.

Neurometabolites have not been extensively researched in EDT and there are no human studies that have examined in detail the effects of EDT on neuronal neurometabolism in any specific brain region. However long-term behavioral, immunologic, and neurochemical alterations have been found in primates exposed to adverse early rearing (Mathew et al, 2003). Adverse early rearing in primates has an enduring impact on adult ^{1}H-MRS measures considered reflective of neuronal integrity and metabolism, membrane structure and glial function, and that these alterations occur in the same brain regions implicated in trauma related psychiatric disorders (Mathew et al, 2003). In a ^{1}H-MRS study of Bonnet macaque primates (Macaca radiata), exposed to uncertain requirements for food procurement for a few months at an early age, where later found to have significantly decreased NAA and significantly increased Glu, Gln and ϒ-aminobutyric acid in the ACC (Glx) (Mathew et al, 2003).

In a review Holmes et al (2005) describe the use of rodent models of early developmental emotional experiences. In rats, postnatal maternal separation can produce lasting increases in emotional behavior and stressor-reactivity, together with alterations in various brain neurotransmitter systems implicated in emotionality, including corticotropin-releasing factor, serotonin, norepinephrine, and Glu (Holmes et al, 2005). The findings of these studies demonstrate the pervasive influence of maternal and social environments during sensitive developmental periods and reveal how genetic factors determine how these early life experiences can shape brain and behavior throughout life (Holmes et al, 2005).

 2.3.5 *Genetics of SAD within the context of EDT*
Despite the significant amount of work and sophisticated technology, it has not been fully elucidated which genes, regions of DNA or which types of genetic changes, alone or in combination, can represent reliable genetic markers of anxiety (Lacerda-Pinheiro et al, 2014). Over the past 15 years, there has been a search for the genetic basis of SAD (Stein & Gelernter, 2010; 2014). Few studies, however, have attempted to disentangle the environmental and genetic determinants of familial aggregation in SAD (Bögels, et al, 2010). Early studies did not demonstrate a specific familial-genetic profile for SAD as compared

with other anxiety disorders (Hettema Neale, & Kendler, 2001; Bögels, et al, 2010). In addition there are few studies that have demonstrated specific genetic transmissions in SAD, within families, independent of the genetic contributions for the broader liability towards mood and anxiety disorders (Bögels, et al, 2010). However more recent research has begun to suggest that there are underlying endophenotypes that may be familial (Stein & Gelernter, 2014). These underlying endophenotypes may be seen as the heritable biological characteristics of SAD. This section on the genetics of SAD will attempt to elaborate on the current genetic underpinnings of SAD within the context of EDT.

Traumatic stress throughout an individual's life, in conjunction with multi-genetic predispositions, is recognized as an important etiological contributor to the development of anxiety disorders (Harvey and Shahid, 2012). It can be said that SAD has a moderate familial basis with regards to genetic heritability, even though specific genetic transmissions have not been established (Stein, Chartier & Hazen, 1998; Stein Chartier, Lizak & Jang, 2001; Stein & Gelernter, 2014). A considerable body of research has attempted to examine the familial aggregation of SAD and shyness (Bögels, et al, 2010). Most of these studies suggest that the contribution of genes to the excess familiality of SAD in monozygotic over dizygotic pairs is modest (20-40%), and similar to many other mental disorders (Norrholm & Ressler, 2009; Stein & Gelernter, 2014). In order to better understand genetic contributions and the specifics of genetic inheritance, genetic linkage studies may be undertaken.

Genetic linkage studies are aimed at identifying specific heritable genes that aggregate along specific loci on a chromosome and are likely to be inherited together during cellular meiosis (Stein & Gelernter, 2014). Genes nearer to each other are less likely to be separated onto different chromatids during chromosomal crossover and are therefore less likely to be considered genetically linked (Strachan, 2010). Gelernter, Page, Stein & Morgan (2004), conducted a genome wide linkage study of SAD with analysis at 10cM (CentiMorgan) resolution in 160 participants, and identified a candidate gene in the SLC6A2 region, which is the norepinephrine transporter protein locus, to be significantly associated with SAD. Follow up research has confirmed the SLC6A2 as a possible genetic susceptibility marker in SAD (Buttenschon et al, 2011). In a study of the $SIRT_1$ gene, involving genotyping 14 SNPs across the gene, of 3429 Swiss men and women with DSM-IV diagnoses, found significant associations between SNPs in SIRT1 and SAD (rs12778366) (Libert et al, 2011). There are

very few genetic linkage studies in SAD and many of the research studies on the genetic basis of SAD has rather examined genetic association studies.

There are, however, still few genetic association studies in SAD (Hamilton, 2009; Stein & Gelernter, 2014). Genetic association studies examine the various genes associated with anxiety related traits in SAD. Domschke et al (2009) found an association between the variant SLC25531 in 5HTTLPR gene and a tendency to blush. Less functionally active 5HTTLPR genotypes are associated with more blushing behavior in SAD compared with controls (Domschke et al, 2009). Sensory processing sensitivities are also an important heritable component of shyness and therefore the development of social fears and social anxiety disorder (Hoffman & Bitran, 2007). The 5HTTLPR gene is also associated with the trait shyness (Arbelle et al, 2003).

Behavioural inhibition (BI) has been considered a temperamental predisposition towards withdrawal, avoidance, fear of the unfamiliar and hyperarousal of the sympathetic nervous system (Hirshfeld-Becker et al, 2008) and BI has been associated with SAD pathophysiology, as an intermediate phenotype (Smoller et al, 2009; Stein & Gelernter, 2014). In a family based association study researchers observed a strong association between childhood BI and several SNPs spanning RGS2 (Smoller et al, 2008). An important finding of this study was that variation in the RGS2 gene was also associated with healthy adults with increased amygdala reactivity to emotional face tasks (Smoller et al, 2008).

Genes that code for amygdala attenuation and functional activation in SAD have received greater attention in attempts to understand the role specific genes have on the mechanisms in SAD. Two important genes under investigation in this study are the tryptophan hydroxylase two (TPH2) and the regulators of G-protein signaling two (RGS2) genes. There are two independent serotonin systems in vertebrates peripheral and central nervous system of which are modulated by TPH1 and TPH2, respectively (Walther and Bader, 2003; Walther et al., 2003). TPH2 is expressed exclusively in the neurons of the Raphe_nuclei in the brainstem (Gutknecht et al., 2007; 2009; Walther and Bader, 2003; Walther et al., 2003) and in myenteric neurons in the gut (Neal et al., 2009), but not in other peripheral organs. This discovery triggered genetic studies on polymorphisms in the TPH2 gene to assess the implications of central serotonin in the development of neuropsychiatric disorders.

Research in imaging genetics in the anxiety disorders is an expanding field and has become a central focus in the clinical neurosciences and psychiatry (Domschke & Dannlowski, 2010). Imaging genetics now plays a central role in understanding how genes code for functional activation in the amygdala (Stein & Gelernter, 2014). However it must be noted that there are some notable shortcomings in the imaging genetics literature, in their methodology, especially with regards to some of the clinically defined neurobiological heterogeneous categorical phenotypes and endophenotypes (Domschke & Dannlowski, 2010).

The principal objective of imaging genetics is to investigate the genetic underpinnings of endophenotypic expression; for example the effects of the serotonin transporter gene (5-HTTLPR) on amygdala activity in response to angry and emotional faces in anxiety disorders (Hariri et al., 2002; Domschke & Dannlowski, 2010). 5-HTTLPR has been a principle focus of many imaging genetic studies in SAD (Lochner et al, 2007; Stein & Gelernter; Hariri et al., 2002; Domschke & Dannlowski, 2010; Samochowiec et al, 2004). For example the 5HTTLPR gene has been associated with shyness in a sample of 98 Israeli children (Arbelle et al., 2003), however this study has not been well replicated in more recent research in SAD (Stein & Gelernter 2014). Beside the 5-HTTLPR gene there are other genes of interest that may also affect amygdala reactivity in SAD (Stein & Gelernter, 2014). Even though the serotonin transporter has been investigated in a number of studies in SAD (Stein & Gelernter, 2014; Kennedy et al., 2001; Stein et al., 1998), its contribution to SAD pathophysiology is currently still questionable (Stein & Gelernter, 2014).

Brown et al. (2005) found that a SNP in the regulatory region of the human TPH2 gene affects amygdala functioning; specifically, the T allele of the common promoter polymorphism [G(−844)T] was associated with relatively exaggerated amygdala reactivity in comparison to the G allele (Brown et al., 2005). The findings of this research provides further insight into the biological significance of TPH2 in the human central nervous system, and have furnished a critical next step in the understanding of the importance the second tryptophan hydroxylase isoform in understanding SAD pathophysiology. In this way, the initial identification of a systems-level effect of a specific polymorphism provides impetus for the subsequent characterization of its functional effects at the molecular and cellular level. Building on findings from imaging genetics (and a subsequent replication; Canli, Congdon, Gutknecht, Constable, & Lesch, 2005; Canli & Lesch, 2007), a recent molecular study has demonstrated that the G(-844)T is in strong linkage with another promoter SNP that affects

the transcriptional regulation of TPH2 and may affect enzyme availability and 5-HT biosynthesis (Chen, Vallender, & Miller, 2008). Furmark et al (2013) examined PET brain activity during a stressful public speaking task over an eight-week period and found 5-HTTLPR and the G-703T polymorphism in the TPH2 gene placebo response was accompanied by reduced stress-related activity in the amygdala. However, attenuated amygdala activity was demonstrable only in participants who were homozygous for the long allele of the 5-HTTLPR or the G variant of the TPH2 polymorphism, and not in carriers of short or T alleles. They also found that the TPH2 polymorphism was a significant predictor of clinical placebo response, and interestingly homozygosis for the G allele was associated with greater improvement in anxiety symptoms with regard to psychopharmacological treatment (Furmark et al, 2013).

EDT such as abuse from a caregiver, can be predictable or unpredictable, each resulting in increased prevalence and severity of a unique set of disorders. Sarro et al, (2014) examined the influence of EDT on both the behavioral expression of adult anxiety and gene expression within the amygdala. Neonatal rats were exposed to unpaired odor-shock conditioning for 5 days, which produces deficits in adult behavior and amygdala dysfunction. Microarray analysis of rats exposed to EDT revealed over-represented genes related to learning and memory, synaptic transmission and trans-membrane transport (Sarro et al, 2014). Gene ontology and pathway analysis identified highly represented disease states related to anxiety phenotypes, including psychiatric disorders such as SAD and PTSD, amongst others. Unpredictable shock during early development increased anxiety-like behaviors in adulthood with concomitant changes in genes related to neurotransmission, resulting in gene expression patterns similar to anxiety-related psychiatric disorders (Sarro et al, 2014).

Notably, the genetic contribution in adult individuals with SAD is approximately half than that in younger patients, with a higher contribution of non-shared environmental influences and less relevance for shared environmental factors (Scaini et al, 2014). This was observed by Scaini et al, (2014), who examined 13 cohorts (42,585 subjects) in 3 meta-analytic estimates of the standardized variance components of aetiological influences on SAD. The proportions of variance accounted for by genetic and environmental factors were calculated by averaging estimates among studies. Meta-analytic estimations showed that genetic and non-shared environmental factors explain most of the individual differences for SAD and social anxiety symptoms (Scaini et al, 2014).

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# 3. METHODS

## 3.1 *Research design*

A quasi-experimental control group design was chosen for the study using a cross-sectional, descriptive, quantitative framework; comparing two case groups and one control group. The sample consisted of 120 participants, selected by non-probability convenience sampling methods. Participants where recruited via internet advertising on a website (www.socialanxiety.co.za), through a radio advert, advertising on various bulletins at the University of Stellenbosch and liaison with other institutions and private psychologists and psychiatrists.

## 3.2 *Sample*

Approximately 265 participants were screened; of these 120 participants were included and assessed. Not all 120 participants were included in the neuroimaging and genetics data analysis, due to post processing and imaging errors and problems encountered during data analysis. Desmond and Glover (2002) in their study of estimation of statistical power in fMRI and MRI suggested that for a liberal threshold of .05, about 12 subjects are required per group in a neuroimaging study to achieve approximately 80% power. Most of the imaging arms of the study included more than 20 participants, which is above the average number for imaging in SAD (Freitas-Ferrari et al., 2010)

The 120 participants were categorized into 3 groups: 37 subjects with SAD with EDT, 32 subjects with SAD without EDT and 31 age, gender, ethnicity, language, handedness and education matched control participants.  Participants were all older than 21 years and were all fluent in English. There was no need for a translator during the interviews and data collection process. Careful attention was paid to the matching of participants on demographic variables.

Participants were screened with the Mini International Neuropsychiatric Interview (MINI) version 6.0 (Sheehan et al., 1998; Lecrubier et al, 1997) to determine whether they met inclusion criteria. Both right-handed and left-handed participants were included and carefully matched across groups. Left-handed participants were also accounted for in all statistical analyses. Other exclusion criteria comprised: all DSM-IV psychotic disorders, bipolar mood

disorders, obsessive-compulsive disorder, eating disorders and current alcohol or substance abuse or addiction disorders. Other Axis one disorders were only included if these disorders werein remission or if they were not the primary disorder on the MINI. All neurological disorders, including head injuries with a loss of consciousness, were exclusions. Other exclusion criteria comprised drug abuse/dependence or alcohol abuse/dependence within the past 6 months (Cooney et al., 2006), and current use of psychotropic medication (except for current SSRI treatment, which they were asked not to take before their MRI scan). Participants were excluded from MRI scanning if they had a cardiac pacemaker, metal prosthesis or pin(s), clips on blood vessels, inner ear prosthesis, an infusion pump, a metal intra-uterine contraceptive device or they were currently pregnant. All metal objects and jewellery were removed before any scanning was undertaken.

### 3.3 *Test instruments and assessments*

### 3.3.1 *Behavioural measures/instruments*

*(i) Liebowitz Social Anxiety Scale (LSAS)*

The LSAS consists of 24 items designed to assess SAD. It consists of two components: a fear rating and an avoidance rating. Each item may be rated on both fear and avoidance in terms of severity, using a scoring scale of between 0-3 (Liebowitz, 1987; Baker et al., 2001). The LSAS may be used to discriminate generalized SAD from SAD with a specific fear focus (Johnson et al., 1996).

*(ii) Childhood Trauma Questionnaire – Short Form*

The Childhood Trauma Questionnaire Short Form (referred to interchangeably in this study as either the CTQ-SF or CTQ) is a self-report measure of the frequency of abuse and neglect in childhood and adolescence (Bernstein et al., 1994; Bernstein & Fink, 1998). The CTQ-SF consists of 28 questions, on a five point Likert Scale. Each question assesses the degree of agreement to a specific aspect or factor of developmental trauma. The CTQ-SF is self-administered and takes approximately 5-10 minutes to complete. The CTQ-SF assesses five dimensions of childhood maltreatment: physical abuse, emotional abuse, sexual abuse, physical neglect and emotional neglect. There are five items on each scale of these scales, plus an additional three-item minimization/denial scale (Thombs et al., 2007). Bernstein et al. (2003) reported good internal consistency of the CTQ-SF for each of the abuse scales across four heterogeneous samples.

The validity index is also used to report possible underreporting of maltreatment (Bernstein et al., 2003). In a study examining substance abuse in adult out-patients and in adolescent psychiatric inpatients, the reliability of the CTQ-SF sub-scales ranged from α = .61 to α = .95 (Bernstein, Ahluvalia, Pogge, & Handelsman, 1997; Bernstein et al., 2003). Each of the 28 items either measures the occurrence of abuse or neglect on a 5 point Likert scale ragning from '1 = never true' to '5 = very true'. Scores on the CTQ-SF range from 5 to 25 for each subscale for a total of 125 for the full CTQ-SF. Higher scores indicate more severe levels of childhood abuse or neglect (score of 25-31 = no trauma, score of 41-51 = low to moderate, 56-68 = moderate to severe, and 73-125 = severe to extreme).

*(iii) Mini International Neuropsychiatric Interview (MINI, version 6.0.0, English, 2008)*
The MINI is used to determine various forms of psychopathology. It is a semi-structured clinical interview, systematized to assess various symptoms in order to determine the diagnostic criteria and presence of psychopathology (Sheehan et al., 1998). The MINI is compatible with both the DSM – IV diagnostic criteria and ICD - 10 diagnostic criteria for mental disorders.  The MINI covers 17 Axis I diagnostic categories of the DSM – IV (Pinninti et al., 2002). It can be administered within approximately 20 minutes. Lecrubier et al. (1997) found good inter-rater and test-retest reliability. Marques and Zuardi (2007), found in their study of general practitioners use of the MINI, that Kappa coefficients ranged from between 0.65 and 0.85, sensitivity was between 0.75 and 0.92, specificity was between 0.90 and 0.99, positive predictive value was between 0.60 and 0.86, negative predictive value was between 0.92 and 0.99, and accuracy was between 0.88 and 0.98.

3.3.2 *Neurocognitive battery*
The array of tests in the neurocognitive test battery were selected to assess verbal memory, fine motor and executive functioning of all participants. The battery included:

*(i) Test for Verbal Fluency*
FAS Words in a minute is a neuropsychological screening test used to determine if the speed and ease of verbal production (Lezak at al, 2012). The typical word fluency task consists of an individual generating a list of words in 60 seconds. Participants are asked to generate lists of words with the letter F, A and S, individually and in a 60 second time frame (Lezak et al, 2012).

*(ii) Weschler Memory Scales III (WMS III)*

The WMS III is a neuropsychological test of learning and memory. It assesses both auditory and visual memory, including the temporal aspects of memory. The WMS III consists of a core battery of six memory tests, which calculate various memory indices. The core memory tests generate eight indices of memory that include: Auditory Immediate, Visual Immediate, Immediate Memory, Auditory Delayed, Visual Delayed, Auditory Recognition, Delayed, General Memory and Working Memory. Reliability coefficients are good and range from .82 to .93.for the WMS III (Lezak et al, 2012).

*(iii) Hopkins Verbal Learning Test (HVLT)*

The HVLT is a test of verbal learning. It consists of 12 words, divided into three semantic categories (thus four words per category). There are also a total of three learning trials, followed by a 24 word recognition list containing all 12 of the original words, plus six semantically related foils and six unrelated word foils (Lezak et al, 2012). There is also a 20-25 minute delayed recall. Scoring yields a total acquisition score, a measure of retention, learning, recall recognition and delayed recognition (Lezak et al, 2012).

*(iv) Wisconsin Card Sorting Test (WCST)*

The WCST was developed to assess abstract behavior and "shift in set" (Lezak et al, 2012). The participant is given between 64 to 128 cards, on which are printed four symbols – a triangle, star, cross or circle – in four colors – red, green, blue or yellow. No two cards are identical. The task requires the participant to place the cards one at time under one of four stimulus cards (Lezak et al, 2012). The participant has to deduce from the examiner the pattern of the specific placement order of the cards. The placement of cards is set by one of three specific target stimuli on each card, such as color, symbol and the number of symbols on each card. The examiner simply answers right or wrong for each placement, without explicitly stating the target stimuli. After a number of placements the participant figures out the set. This continues until six runs of ten correct placements have been made (Lezak et al, 2012).

*(v) Stroop Color Word Test (SCWT)*

The Stroop Colour Word Test is a test of attentional processing and flexibility. The task examines one's ability to read words more quickly and automatically than one's ability to

name colors, for example a word is printed or displayed in a color different from the color it actually names. The individual has to read the colour in one task and name the colour perceived without reading it in another task. Ability to dual track and shift attention is scored (Lezak et al, 2012).

*(vi) Halstead Category Test (HCT)*

The HCT distinguishes individuals with brain damage from normal individuals. The HCT contains 208 visual stimuli that assess conceptual and spatial reasoning. Participants are asked to figure out the principle presented in each of the visual stimuli. They are asked to choose one of four possible responses listed from I to IV on the test answer sheet. The test may also be timed and a set of scores generated for both accuracy and time to completion (Lezak et al, 2012). The test should normally take approximately 15 minutes to complete.

*(vii) Trail Making Test (TMT)*

The TMT is often used in conjunction with other tests of orientation and attention. It is primarily used as a test of scanning, visuomotor tracking, divided attention, and cognitive flexibility (Lezak et al, 2012). Participants have to connect consecutive numbers on a worksheet (form A) and then connect consecutive numbers and letters in order of increasing sequence and alphabetical order in alternation of sequence on a second worksheet (form B).

*(viii) Grooved Pegboard*

The grooved pegboard is a cognitive-perceptual motor test (Lezak et al, 2012). The test consists of a board containing a 5 x 5 set of holes, which are grooved and the grooves are angled in different directions. A metal peg is inserted into each whole, one at a time. Each peg has a ridge, so that it has to be rotated into the correct position in order to fit into the grooved hole. A score is computed for time to completion and motor speed per second. Both hands are tested, and scores for each are also compared.

*(ix) Edinburgh Handedness Questionnaire*

The Edinburgh Handedness Questionnaire consists of a set of 20 items, used to quantitatively and objectively assess handedness (Oldfield, 1970). Scores are computed for subjects and will indicate whether they are either left handed, right handed or ambidextrous.

### 3.3.3 *Neurocognitive testing format*

Neurocognitive tests were administered in a set format for all participants. A registered clinical psychologist undertook the administration. The majority of testing was done by the principal investigator (David Rosenstein). Some tests that assessed verbal memory were alternated, in order to more accurately assess delayed recall without conflicting cognitive processing on tasks (Lezak, 2012). The tests were administered in the following order:

1) WMS III
2) WCST
3) Test for Verbal Fluency Computer Assisted
4) HVLT
5) SCWT
6) HCT
7) TMT
8) Grooved Peg Board
9) Edinburgh Handedness Questionnaire

### 3.4 *Neuroimaging*

The Siemens 3 Tesla Allegra fMRI scanner at CUBIC (Tygerberg) was used to acquire images. ^{1}H-MRS data was collected in order to elucidate and evaluate neurophysiological activation (of the amygdala specifically). Some studies have reported decreased rather than increased amygdala activation in PTSD (Etkin and Wager, 2007). The extent to which these differences between SAD and PTSD may be mediated by early developmental trauma in adults with SAD has not been elucidated and, as such, formed the focus of this study.

### 3.4.1 *Structural Magnetic Resonance Imaging (sMRI)*

sMRI was used to ascertain structural and volumetric (morphometric) data of specific target brain regions of the limbic system and associated neurocircuitry across the three study groups. A high resolution T1-weighted 3D MPRAGE structural image was first obtained with the following parameters: sagittal orientation, TR =2300ms, TE = 3.93ms, FOV = 220mm, 160 slices; 256x256 matrix; 1x1x1mm^3 resolution; 9 minute scan time.

3.4.2 *Proton Magnetic Resonance Spectroscopy (H[1]-MRS)*

Single voxel proton magnetic resonance imaging is a neuroimaging (H[1]-MRS) technique that allows for the investigation of neurometabolite profiles of the human brain. H[1]-MRS generates magnetic resonance signals from hydrogen nuclei (protons) of neurochemical molecules. This produces a resonance spectrum with peaks that are unique to each molecule. This approach is aimed at better understanding the neurobiology of these disorders and improving treatment strategies. In anxiety disorders, specifically, H[1]-MRS allows for in vivo quantification of various neurometabolites (e.g., *N*-acetylaspartate (NAA), *N*-acetylaspartylglutamate (NAAG), creatine, phosphocreatine, choline, myoinositol) in brain regions central to their pathophysiology. For example, *N*-acetylaspartate, which is the most prominent H[1]-MRS signal, is a marker of neuronal integrity. H[1]-MRS was performed in order to evaluate the neurometabolite profile of the amygdala, specifically glutamate across the three groups. A 10x12x12 Voxel was placed within the left amygdala with the following reference vectors: position: L16.5 P6.3 F7.5 [mm]; orientation: C > S33.2 > T-28.4 and rotation: -54 degrees. Functional activations during social fear tasks have been found to be more consistent in the left compared to the right amygdala (Hardee et al., 2008; Baas et al., 2004). In addition the left amygdala has greater activation when individuals are tasked with distinguishing between fearful eyes and gaze shifts compared with the right amygdala. Left amygdala activation also correlated with greater fear discrimination (Hardee et al., 2008). In a systematic review and meta-analysis combining results from 54 fMRI and PET studies, Baas et al. (2004) found a common pattern of lateralized amygdala activation; the left amygdala was more often activated than the right amygdala, suggesting different roles for the left and right amygdala in emotional processing. Further analysis showed that the predominant left amygdala activation was not significantly related to stimulus type, task instructions, differential habituation rates of the left and right amygdala, or the elaborateness of information processing. The following scan parameters were used: TR 2200ms TE 30ms, 544 Averages at HE coil, Vector size was 512. Full water saturation was set after a pre-set automatic shimming with a following 5 minutes manual shimming. The total scan time for the H[1]-MRS was 21 minutes.

3.5 *Genotyping*

Blood samples for DNA were drawn at the first study visit by venous puncture and collected in two 10ml ethylene-diamine-tetra-acetic acid (EDTA) tubes. 10-20 ml of blood were collected for analysis for each sample.  Blood samples were sent to the research laboratory

within 24 hours of sampling. DNA was extracted from venous blood (10 ml) using phenol/chloroform extraction, and each sample was then genotyped for polymorphisms in the *TPH-2* and *RGS2* genes.

*(i) TPH-2*

A common promoter polymorphism (T-703G or rs4570625) was investigated in the TPH-2 gene. It has been predicted that this SNP contains a base change that affects the binding of transcription factors, and may, therefore, have the ability to modulate TPH-2 gene expression (Lin et al., 2007). The SNP was genotyped using a pre-designed, commercially available Taqman® 5' nuclease assay (Applied Biosystems, Foster City, California; www.allsnps.com). The PCR reactions were performed in a total volume of 5ul, using 384-well plates, and were amplified in a GeneAmp® PCR system 9700 (Applied Biosystems) under the following conditions: 50 ºC for 2min; 95 ºC for 10 min; 40 cycles of 92 ºC for 15s, followed by 60 ºC for 1.5 min. The fluorescent intensity of the final reaction product was measured using the 7900HT Fast Real-Time PCR System (Applied Biosystems) and analysed with Sequence Detection Systems version 2.3 (Applied Biosystems).

*(ii) RGS2*

A SNP occurring in the 3ÚTR of the RGS2 gene was investigated. This SNP has been found to be associated with anxiety-related temperament (Yalcin et al., 2004), and is thought to be functional since the G-allele is associated with reduced *RGS2* expression (Semplicini et al., 2006). The SNP was genotyped using a pre-designed, commercially available Taqman® 5' nuclease assay (Applied Biosystems, Foster City, California; www.allsnps.com), as described above.

**Clinical factors**

Diagnostic screening for psychopathology and SAD specifically. Assessments for EDT and grouping into SAD with EDT, SAD without EDT and healthy controls

**sMRI**

sMRI of limbic system volumes including GAT analysis of total brain grey matter volumes

**Neurocognition**

Neurocognitive testing of memory, executive functioning and motor function

**$_1$H-MRS**

$_1$H-MRS of neurometabolites in the left amygdaloid complex

**Genotyping**

Genotyping RGS2 and TPH2 Polymorphisms for further analysis of SAD mechanisms in amygdala specifically

**Figure 3** demonstrates the various data collection points, which include clinical and demographic factors, assessed by questionnaires, neuroimaging components including sMRI and $^1$H-MRS, neurocognitive testing and genotyping. These various data points were used collectively toexplore many of the complex and inter-related mechanisms in SAD within the context of EDT.

3.6 *Procedures*

3.6.1 *First study visit*

Each participant had approximately three study visits. The first study visit consisted of obtaining informed consent, screening, a diagnostic assessment and blood draw. After the study procedures had been explained and consent given, each participant was screened for age, gender, educational background, use of any psychotropic medication or other medication, previous head injury, previous episode of loss of consciousness, and known medical, neurological and psychiatric conditions. All 120 participants were then assessed for SAD and other psychopathology, using the Mini International Neuropsychiatric Interview (MINI). All 120 participants were further screened for SAD, using the self-report Liebowitz Social Anxiety Scale (LSAS). Cut off scores of =>60 on the LSAS were used to determine whether a participant qualified for SAD in the study (Safren et al., 1999). EDT was recorded using quantitative and qualitative self-report measurements with the CTQ (Thombs et al., 2007) and the CTI (Fink et al., 1995). Both are retrospective measures of childhood abuse. To clearly distinguish between individuals with EDT of versus non-traumatized individuals the interpretation guidelines suggested by Bernstein & Fink (1998) were used. The CTQ-SF cut-scores are divided into 4 categories; 'none to minimal trauma' (scores 25-36), 'low to moderate trauma' (scores 41-51), 'moderate to severe trauma' (scores 56- 68) and 'severe to extreme trauma' (scores 73-125) (Bernstein et al., 1997; Bernstein & Fink, 1998). Even though exact figures of childhood traumatisation are not available for the South African population, higher exposures to childhood trauma can be expected in South Africa in comparison to western civilizations due to its records of violence, its political history and socioeconomic status (Seedat, Van Niekerk, Jewkes, Suffla, & Ratele, 2009; ). It was therefore decided to classify individuals in this study as not having EDT with  a score of <40 and individuals having EDT with scores ranging from 46 to 125. Further individuals with scores between 41 (lower score limit for the 'low to moderate trauma' category)  and 46 (mid-score for the 'low to moderate trauma' range) were excluded due to more clearly distinguish between individuals with EDT and individuals not having EDT, in this study (Bernstein et al., 1997). These findings are based on individuals qualifying with at least moderate levels of childhood trauma to be included in the study (Berinsetin & Fink, 1998; Bernstein et al, 2003). Blood sampling was then used to examine the following genes: *TPH-2* and *RGS2 (see above re: genotyping and blood draw analyses)*. These genes were extracted to evaluate genetic vulnerability markers for SAD. All participants' blood was stored at the

MAGIC laboratory at the Department of Biomedical Sciences, Faculty of Health Sciences, Tygerberg Campus.Venous blood was drawn from *consenting* participants by the study nurse.

### 3.6.2 *Second study visit*

At the second visit, participants underwent neurocognitive assessment (out of the MRI scanner) for verbal fluency, memory, fine motor performnce and executive functioning. This was accomplished using the neurocognitive battery mentioned above. Neurocognitive screening took approximately 2 hours per participant.

### 3.6.3 *Third study visit*

At the third visit, participants underwent sMRI and ^{1}H-MRS scanning. Participants underwent preparatory training in sMRI and ^{1}H-MRS procedures. This included a brief description of how the modalities work and the necessary precautions that need to be taken for scanning. Scanning took approximately 1 hour per participant. Computer literacy was not a requirement, as many of the neurocognitive tests and assessments that were administered required minimal or no computer literacy. The computer interface for the assessments and neurocognitive tests were basic and easy to use for individuals with and without computer knowledge.

```
┌─────────────────────────────────────┐
│ 1) Advertising and recruitmnent     │
│               and                   │
│        Telephonic screening         │
│ 2) Project information and research │
│ brief given to potential participants│
│             telephonically          │
└─────────────────────────────────────┘
                    │
┌─────────────────────────────────────┐
│          Intitial screening         │
│ 1) Biographical information         │
│ 2) Diagnosti assessments:           │
│        MINI, LSAS                    │
│ 3) Trauma assessment (CTQ)          │
│ 4) Other self-report measures       │
└─────────────────────────────────────┘
```

| Exclusion of participants who do not meet eligibility criteria | Group with SAD and EDT | Group with SAD without EDT | Control Group |
|---|---|---|---|
| | Neurocognitive assessments and genotyping | Neurocognitive assessments and genotyping | Neurocognitive assessments and genotyping |
| | sMRI and $^1$H-MRS | sMRI and $^1$H-MRS | sMRI and $^1$H-MRS |

**Figure 4** presents the study flow diagram indicating the three study visits and data collection procedures contained in each study visit. Exclusion criteria are also loosely explained in the flow diagram.

3.7 *Investigator roles*

The principal investigator (David Rosenstein) was responsible for the recruitment of participants, matching of the sample, initial screening of participants, neurocognitive testing of participants, statistical analysis and interpretation and write up of the results in collaboration with other investigators. Dr Sian Hemmings provided oversight of the genetics sub-study and assisted with the statistical analysis of genetics data. Fatima Ahmed assisted with fMRI and structural data analysis and Dr Aaron Hess assisted with the $H^1$-MRS protocol design, implementation and data analysis. Prof Soraya Seedat was the overall principal supervisor for the research project and Prof Christine Lochner was co-supervisor for the research project. All tests where administered in either English or Afrikaans. A Xhosa-speaking interpreter was available to assist, however the interpreter was not required for the administration of any of the tests and questionnaires. Research assistants Melanie Deist and Melanie Bishop were employed to help with administration of some of the tests. Dr Jonathan Zwart, an astrophysicist and expert in Bayesian statistical analysis assisted with the Bayesian statistical analysis.

3.8 *Data analysis*

3.8.1 *Statistical Methods*

3.8.1.1  *A Bayesian approach*

The popularization of the use of Bayes' theorem in recent years has revolutionized the science in fields as diverse as cosmology, biology, ecology, neuroscience and psychiatry. Its self-consistent philosophy naturally embodies the scientific method: one has an initial belief, updates that belief by carrying out an objective experiment, gains quantifiable information because of that, and updates one's belief before beginning the next experiment (Kruscke, 2013). One thus does away with the `frequentist' approach in which either an ensemble of imaginary experiments is considered or where there is an arbitrary (but conventional) choice of `p-value'.

Masson (2011) sets out the advantages of the bayesian approach. These advantages are not simply philosophical but include the treatment of data from small sets or samples, with the benefit that the data does not have to fit a gausian or normal distribution. Now consider the current problem of hypothesis testing: The null hypothesis, that a mean NM concentration is the same for the control and SAD (or SAD with EDT) groups, or that the latter has a greater mean than that of the control group. Stating these,

$H_0$: P(mean SAD > mean control|SAD data,control data,I) [1]

$H_1$: P(mean SAD = mean control|SAD data,control data,I) [2].

Following Sivia (2006), Bayes' theorem can be written for hypothesis H_i as

P($H_i$|D,I) = L(D|$H_i$,I) . PI($H_i$|I) / Z(D|I), [3]

where H_i is the ith hypothesis, D is all the experimental data, and I are any explicit or implicit assumptions. P is the posterior probability of the hypothesis that we seek, the likelihood L encompasses the experimental data for a given hypothesis, the priors PI on the hypotheses are any prior knowledge or perhaps controversial prejudices about the hypotheses, and Z is the quantifiable evidence (also in the colloquial sense) of the given data.

Now taking the ratio of [3] for [1] and [2], the evidence for each model cancels out to give the desired `odds ratio':

P($H_1$|D) / P($H_0$|D) = L(D|$H_1$) / L(D|$H_0$) . PI($H_1$) / pi($H_0$) [4]

Where we have dropped the I's solely for clarity, PI($H_1$) / PI($H_0$) represents the ratio of the prior probabilities for the two hypotheses. These could be drawn from the results of a previous experiment, but since these are typically not available we assume equiprobable prior probabilities, i.e. pi($H_1$) = pi($H_0$), which is equivalent to taking our experiment or investigation in this study to be novel and independent, where the posterior odd ratio is equal to the likelihood ratio. We note that our posterior ratios will be available to others for input into their analyses as suitable prior ratios on hypotheses. Updating the 'belief' or knowledge about the original hypothesis is an advantage of Bayesian statistics.

L(D|$H_i$) represents the data given a particular hypothesis. We evaluate this numerically using the BEST MCMC algorithm (Kruschke, 2013), effectively simulating 2000 datasets for each hypothesis and comparing their relative probabilities. For more details on the BEST algorithm we refer the reader to www.indiana.edu/~kruschke/BEST. www.sumsar.net/best_online gives a way to run the hypothesis testing online, though we used the python version pyBEST available from https://github.com/strawlab/best. We ran the analysis separately for each experiment for each hypothesis in turn, and in each case for each of the groups under investigation, in both group wise comparisons and in correlation

92

equivalent testing.

Having evaluated the likelihood ratio, the odds ratio was obtained. This is simply the relative probability of the two hypotheses given the data. This is in contrast to the binary, frequentist approach whereby one hypothesis is rejected absolutely while the other is accepted with some probability threshold, although a direct comparison can be undertaken by thresholding the bayesian odds ratios.

The overall statistical model was based on the difference between the groups (SAD with EDT, SAD without EDT and controls) on the various parameters under observation ($H_1:H_0$). A mixture of statistical methods was chosen for the data analysis. However the study predominantly used a Bayesian approach to test probabilities rather than standard null-hypothesis significance testing (NHST). There are a number of theoretical and philosophical reasons for using a Bayesian approach rather than a NHST approach for statistical analysis. These included the following: 1) In NHST the p values generated may be misleading, in that they fail provide the information that a researcher actually wants to have (Masson, 2011). The p value is a conditional probability that indicates the likelihood of an observed result given that the null hypothesis is correct: $p(D|H_0)$. A p value is a binary value that has arbitrary significance, in that a researcher may accept $H_0$ based on the p value selection of 0.5, were $H_0$ may not be correct. 2) Bayesian data analysis avoids the muddle of fixed versus random effects, treating all effects as random. The importance of this is that it can be theoretically difficult to adequately discriminate between the various factors and effects that contribute to the model; it can also be very difficult to account for all effects in the model, which is often assumed by many NHST approaches. 3) Bayesian testing provides the estimates of effects and the variance of effects with corresponding uncertainty, which traditional NHST testing often does not provide, such as in the traditional $t$ test (Kruschke, 2013). 4) Difficulties with normality and distribution, as well as small sample sizes and missing values are easily handled in this Bayesian framework (Chakraborty et al, 2012).

Even though the data obtained in this study contains a larger n than other sMRI and ^{1}H-MRS studies of SAD it was decided to use Bayesian statistical approaches due to the robust power and utility in providing a rich data set to discuss the various findings in the study. The Bayesain outputs allow for a descriptive and broader approach to discussing statistical

research findings (Kruschke, 2011).

3.8.1.2 *Bayesian data analysis*

The Bayesian analysis examined the probabilities of the likelihood of our data findings (posteriors) given the evidence (priors). Our priors were uninformative, as there is no informative prior knowledge about the specific hypotheses under examination (there is no Bayesian information on sMRI, ^{1}H-MRS, clinical data or genetic data in SAD within the context of EDT). We examined two Bayesian models to compare group differences. The first was Bayesian estimation supersedes the t test (BEST) (Kruscke, 2012, 2013) and the second was Bayesian test for accepting and rejecting the null hypothesis (Rouder et al, 2009). For the analyses in BEST, every sample consisted of a burn-in period of 10 000 iterations or simulated experiments, based on the standardized model parameters suggested by BEST (Kruschke, 2012). The data sample had a normal distribution. The model was checked for convergence by considering the relevant kernel density plots and was also checked diagnostically through the autocorrelation plots within the python BEST program. This showed that throughout the iterative process the autocorrelation was satisfactorily reduced to a nominal amount. In determining the model, uninformative/vague prior parameters were selected, given the relatively novel data and hypotheses being explored. The prior parameters for the model were fixed because of the goodness of fit of data to the model and the fact that the prior parameters were relatively vague. During all runs of the data we made sure that all of the effect estimates converged to a common figure; this was confirmed by examining all output graphs generated by the BEST.

3.8.1.3 *Null hypothesis significance testing (NHST) statistical analysis*

Some data was analyzed using SPSS (Statistical Package for the Social Sciences) version 19.0. Descriptive statistics, analysis of variance (ANOVA), group mean differences and correlations were performed for the 3 groups on socio-demographic, clinical, and neurocognitive variables. Two-tailed bivariate and partial Pearson correlation coefficients were also calculated. For the gene data, we dichotomised genotypes that were compared. The alpha coefficient was set at .05 for all analyses. Multiple regression analysis was used to assess the predictive power of childhood trauma, genotype, behavioral and neurocognitive measures on amygdala responsiveness.

94

### 3.8.2 *Analysis of Genetic Data*

(i) For the TPH2 gene: participants who are T (risk) allele carriers were compared with G allele (GG) homozygotes, and (ii) for the RGS2 gene–rs4606: participants who were G (risk) allele carriers were compared to those who were C allele (CC) homozygotes. The alpha coefficient was set at .05 for all NHST genotype analyses. Correlations were performed on genotype data and sMRI brain volumes.

### 3.8.3 *Neuroimaging analysis*

### 3.8.3.1 *Structural MRI analysis*

Between-group grey matter differences were determined for the amygdala, insula, anterior cingulate cortex and the hippocampus. A high resolution T1-weighted 3D MPRAGE structural image was first obtained with the following parameters: sagittal orientation, TR =2300ms, TE = 3.93ms, FOV = 220mm, 160 slices; 256x256 matrix; 1x1x1mm^3 resolution; 9 minute scan time. sMRI data were analyzed using Freesurfer software.

### 3.8.3.2 *Spectroscopy analysis*

Spectroscopic data were analyzed with the assistance of Dr Aaron Hess, from the University of Cape Town Biomedical Engineering and Oxford University using LCmodel. Significance was set at 0.025 (25% on the LCModel output) in LCModel, for includable neurometabolites. Aaron and David performed post-processing runs each in FSL, in order to derive the best outcome set.

PLOT 1:  Page 1 of test run output
Data of: Biomedizinische NMR Forschungs GmbH, Goettingen

LCModel (Version 6.2-0) Copyright: S.W. Provencher.        Ref.: Magn. Reson. Med. 30:672-679 (1993).        26-August-2007  19:39

| Conc. | %SD | /Cr | Metabolite |
|---|---|---|---|
| 0.227 | 130% | 4.5E-02 | Ala |
| 0.491 | 90% | 9.7E-02 | Asp |
| 5.059 | 4% | 1.000 | Cr |
| 0.398 | 114% | 7.9E-02 | GABA |
| 0.000 | 999% | 0.000 | Glc |
| 1.434 | 48% | 0.283 | Gln |
| 5.049 | 13% | 0.998 | Glu |
| 0.835 | 24% | 0.165 | GPC |
| 0.629 | 32% | 0.124 | PCh |
| 4.362 | 6% | 0.862 | Ins |
| 0.162 | 186% | 3.2E-02 | Lac |
| 6.057 | 4% | 1.197 | NAA |
| 1.195 | 24% | 0.236 | NAAG |
| 6.55E-02 | 120% | 1.3E-02 | Scyllo |
| 1.529 | 30% | 0.302 | Tau |
| 1.091 | 25% | 0.216 | -CrCH2 |
| 0.326 | 77% | 6.5E-02 | Gua |
| 1.464 | 5% | 0.289 | GPC+PCh |
| 7.253 | 4% | 1.434 | NAA+NAAG |
| 6.483 | 13% | 1.282 | Glu+Gln |
| 14.264 | 22% | 2.820 | Lip13a |
| 0.000 | 999% | 0.000 | Lip13b |
| 0.000 | 999% | 0.000 | Lip09 |
| 4.890 | 19% | 0.967 | MM09 |
| 1.320 | 95% | 0.261 | Lip20 |
| 7.925 | 22% | 1.567 | MM20 |
| 1.713 | 35% | 0.339 | MM12 |
| 6.118 | 28% | 1.209 | MM14 |
| 3.459 | 32% | 0.684 | MM17 |
| 14.264 | 22% | 2.820 | Lip13a+Lip13b |
| 22.094 | 16% | 4.368 | MM14+Lip13a+L |
| 4.890 | 19% | 0.967 | MM09+Lip09 |
| 9.245 | 20% | 1.828 | MM20+Lip20 |

NO DIAGNOSTICS

MISCELLANEOUS OUTPUT
FWHM = 0.035 ppm      S/N = 14
Data shift = 0.006 ppm
Ph:  45 deg      -17.4 deg/ppm

INPUT CHANGES
NEACH=0
NAMRAC(1)='NAA'
NAMRAC(2)='NAAG'
hzpppm=84.47
nunfil=2048  deltat=.0005

**Figure 5** is a a sample of $^1$H-MRS spectra output just before post processing with LCModel, showing the ppm spectral line of the various neurometabolites under investigation. Neurometabolite outputs in the right column indicate statistically significant outputs in blue with an SD of 25%. We used a threshold of 20% to select significant neurometabolites for further analysis.

3.9 *Ethical considerations*

The protocol was submitted to Stellenbosch University's Health Research Ethics Committee for approval. Participation was completely voluntary and written informed consent was obtained from all participants prior to their participation in any of the study visits. There were two informed consent forms for the study. The first form was a general consent form and the second form was a genetics and blood draw consent form. Both consent forms were given to participants at the initiation of the study.  The nature of the study, testing and administration of tests and questionnaires was also explained to participants by providing a participant information sheet and answering questions about the research study. Confidentiality was maintained by excluding identifying details (such as name, address or telephone numbers) from all study-related documentation. Anonymity was maintained by de-identifying collected data; participants were identifiable only by a unique identifier code.

There were no incentives for participation, however participants were reimbursed for travel costs at each visit and received a gift voucher of 100 rand at the completion of the last study visit.  All participants had the right to withdraw from the research study at any time, without disadvantaging themselves in any way. Participants could also request feedback on questionnaires and tests once the study was completed. This was done in the form of the completed research report, which provided an overview of scores and results, rather than personalised feedback.

No adverse effects due to the administration of the questionnaires and tests selected in this study or the greater research project were expected although it was possible that some participants could become distressed by the nature of the information requested in the measuring instruments. Follow up telephone numbers for clinical services were made available to participants at the time of consent and upon completion of the questionnaires and tests. If there was any distress caused at any point in the study, we made sure that participants could be referred to their local community clinics and mental health services. They were also assisted with the referral process to ensure that they received adequate support and care.

There is no ionizing radiation emitted from fMRI scanning, therefore no hazardous effects have been reported, aside from occasional yet harmless, heating of the body. It could become uncomfortable lying in the machine for extended periods of time and some individuals with anxiety become anxious in the confines of the fMRI machine. The session in this case was terminated immediately.

Individuals with metal implants (as described above in the sample section under exclusion criteria) may have been at risk, especially if they had any electronic prostheses. Individuals were all therefore made thoroughly aware and assessed before scanning and individuals with metal implants/prostheses were excluded from the study. No follow up counseling or referral was needed due to scanning. There were no incidental findings that were detected on any of the participant scans during the scanning process.

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# 4. NEUROCOGNITIVE FUNCTIONING IN SOCIAL ANXIETY DISORDER IN THE CONTEXT OF EARLY DEVELOPMENTAL TRAUMA: A BAYESIAN ANALYSIS

**This chapter was submitted s a research paper to the BMC Psychiatry journal for publication and has thus been represented as a journal publication chapter. This chapter will report on the neurocognitive findings in SAD within the context of EDT.**

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4.1 *Abstract*

Objective: A number of neurocognitive difficulties have been found in social anxiety disorder (SAD). However, the impact of early developmental trauma (EDT) on neurocognition has not been fully elucidated in SAD. A neurocognitive test battery was administered to determine neurocognitive performance in verbal memory, fine motor performance and executive functioning in participants with SAD and EDT.

Methods: Individuals with SAD and EDT (n = 25), individuals with SAD without EDT (n = 24) and healthy controls (n = 26) matched for education, handedness, gender and age, with a mean age = 32.9 years [SD = 8.9], were compared on cognitive tasks. A Bayesian approach was used to analyze neurocognitive test results.

Results: Significant statistical differences were found in verbal memory, executive functioning and fine motor performance between the SAD with EDT and control groups. No differences in cognitive performance were found between the SAD with EDT and SAD without EDT groups. Verbal memory difficulties were found in the SAD without EDT group compared with controls.

Conclusion: This study is unique in its comparative assessment of the effects of EDT on neurocognition in individuals with SAD. In conclusion the results show that individuals with SAD without EDT have relatively few cognitive difficulties. However, individuals with SAD and EDT group had difficulties in the domains of memory, and psychomotor functioning and executive functioning; suggesting that the combination of SAD and EDT may lead to specific cognitive performance difficulties later in life. Neurocognitive functioning in SAD with and without EDT may be underpinned by different pathobiological processes, which require further investigation in a larger clinical sample.

4.2 Introduction

Social anxiety disorder (SAD) is characterized by fear and avoidance of a number of social situations. Epidemiological surveys indicate that SAD is one of the most common psychiatric disorders with a 12-month prevalence rate of 6.8% and a lifetime prevalence rate of 12.1% (Kessler et al., 2005). According to Stein et al. (2010) the lifetime prevalence of SAD is more common in developed countries (6.1%) than in developing (2.1%) countries. The age of onset for SAD is usually between 12 and 13 years, however it may begin earlier (Bandelow & Stein, 2004; Ollendick, Hirshfield-Becker, 2002). Even though SAD may begin in early adolescence it often has a chronic unremitting course through adulthood and may cause significant impairment (Stein & Stein, 2008). For example, up to 49% of individuals with SAD in an American study left school prematurely; 24% of these individuals attributed this to anxiety (van Ameringen, Mancini & Farvolden, 2003). It has been hypothesized that these school related difficulties may be due to impaired cognition during stressful situations (Graver & White, 2007), and that neurocognitive difficulties represented by certain neuropsychological constructs may underlie SAD (Amir & Bomyea, 2010).  However, unlike the psychotic disorders and mood disorders, there is relatively little data on neurocognitive impairments in anxiety disorders, with the exception of obsessive compulsive disorder and posttraumatic stress disorder (Fujii et al, 2013; Coles, Turk, & Heimberg 2002). Also, few studies have fully explicated neurocognitive functioning in anxiety disorders and SAD specifically (Castaneda et al., 2011; Graver & White, 2007; Sutterby & Bedwell, 2012), especially in representative non-clinical samples and populations (Castaneda et al., 2011).

Empirical evidence supporting neurocognitive underpinnings in SAD (and generalized SAD specifically) show mixed results. The various studies that do present some evidence for neurocognitive dysfunction in SAD point specifically to the domains of memory, executive function and attention (Graver & White, 2007; Fujii et al. 2013). Of these neurocognitive difficulties, memory processes appear to play a substantial and important role in the maintenance of social fears in SAD (Heinrichs & Hoffman, 2004). Airaksinen, Larsson and Forsell (2004) examined various neurocognitive difficulties across a wide range of anxiety disorders that included SAD and found poorer performance on both executive functioning and episodic memory. However, on examination of each separate anxiety disorder, episodic memory difficulties were restricted to the SAD group (Airaksinen et al., 2004). Asmundson, Stein and Larson (1994) found significant memory recall deficits for verbal information processing. Although memory recall difficulties have been reported, there is also evidence for

biased memory processing. For example, Leigh and Hirsch (2011) provided evidence in SAD that worry content is either predominantly verbal or visual. They found that worry content that was predominantly verbal, rather than visual, depleted working memory capacity. The authors also found that the verbal nature of worry contributed to the depletion of working memory resources during worry among 'high-worriers' (Leigh & Hirsch, 2011). Moscovitch et al. (2011) found memory recall biases in SAD when individuals recalled certain autobiographical memories for social experiences associated with greater negative cognitive and emotional consequences. Episodic memory also showed marked degrading in detail compared with controls (Moscovitch et al., 2011).

Memory encoding processes are largely affected by attention, which is also reported to be affected in SAD (Rapee et al, 1997). Attentional biases have been shown in individuals with SAD who demonstrate more attention to threat-relevant words, in various set shifting studies (Lundh & Ost, 1997; Hope, Rapee, Heimberg, & Dombeck, 1990; Maidenberg, Chen, Craske, Bohn, & Bystritsky, 1996; Mattia, Heimberg, & Hope, 1993). Individuals with SAD are less likely to attend to facial expressions compared with individuals without SAD (Rapee et al, 1997; Horley, Williams, Gonsalvez & Gordon, 2003). Further, individuals with SAD have been found to have more impaired verbal attention compared to controls, although spatial attention has been found to be intact (Asmundson, et al., 1994). However, in a study by Cohen et al. (1996), individuals with SAD were found to have lower spatial working memory for attentional processing and set shifting performance than controls. This is supported by findings that spatial span scores in SAD participants are reduced during stress, improved for healthy controls and show no change for SAD with comorbid depression (Graver and White, 2007).

A number of studies (Cohen et al., 1996; Graver and White, 2007) have found executive functioning performance difficulties in SAD.  Executive functioning can be defined as a set of general-purpose regulatory mechanisms of human cognition and action, linked to the prefrontal cortex of the brain (Miyake & Friedman, 2012). Executive functioning encompasses a number of important cognitive processes, including working memory, set shifting or task flexibility in information processing, and planning (Lezak, et al., 2012). Graver and White (2007) found that during stress SAD participants performed more slowly on the Trail Making Test version 'B', an executive functioning test of set shifting, compared with healthy controls and participants with SAD and depression. Further analysis of

normative data for the Wisconsin Card Sorting Test (WCST) indicated that during stress healthy controls and SAD with depression groups improved significantly on the total errors score, while the performance of the SAD group declined (Graver & White, 2007).

SAD may have childhood developmental origins (Ollendick & Hirshfield-Becker, 2002). Individuals with SAD report high rates of childhood trauma (Bandelow et al., 2004; Etkin & Wager, 2007; Safren, Gershuny, Marzol, Otto & Pollack, 2002; Simon et al., 2009; Zayfert, DeViva & Hofmann, 2005). Thus EDT constitutes a significant risk for the development of SAD in later adult life (Kuo et al, 2011). EDT may be broadly defined as any traumatic experience that occurs before the age of 18 years (Bernstein & Fink, 1998). A traumatic event may present itself as sexual abuse, familial violence, separation from parents, parental marital discord, childhood illness, parental neglect, poor parenting or rearing styles, or other traumatic experiences during childhood (Bandelow et al., 2004). Currently there are no studies that have examined the effects of childhood trauma on neurocognitive functioning in SAD. However, there is evidence to suggest that EDT has an effect on cognitive performance in the development of anxiety disorders (Gould et al., 2012). For example, in a recent study by Gould et al. (2012) that examined the effects of childhood trauma on cognitive functioning, deficits on measures of memory and executive and emotional functioning were found.

In the present cross-sectional study, we compared neurocognitive functioning across three groups: SAD with EDT, SAD without EDT and a healthy control group. Given the absence of well-established studies detailing neurocognition in SAD within the context of EDT, we assessed a number of cognitive domains including verbal fluency, memory, fine motor performance and executive functioning. We compared a primary group of participants with DSM-IV generalized SAD with EDT, with SAD without EDT, and a group of healthy control participants to identify neurocognitive differences associated with SAD with EDT compared with SAD without EDT and with controls.

### 4.3 *Methods and materials*

### 4.3.1 Participants

Seventy five adults were recruited through a number of private practices, an online website and an electronic advertisement posted at a local university (Stellenbosch University, Cape Town, South Africa). All participants were 21 years and older.  The healthy controls (*n* =

*26)*, SAD with EDT (*n*= 25), and SAD without EDT (*n* = 24) groups were matched on age, education, gender, ethnic background, and handedness. The mean age of participants was 32.9 years (SD 8.9 years).

### 4.3.2 *Instruments*

All measures were administered in English and all participants completed the following clinical assessments: The Liebowitz Social Anxiety Scale, (LSAS) (Baker, Heinrichs, Kim, & Hofmann, 2002), Mini International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998) and Childhood Trauma Questionnaire Short Form (CTQ-SF) (Bernstein & Fink, 1998). The MINI (version 6.0) is a clinician administered structured diagnostic assessment (Sheehan et al., 1998; Lecrubier et al., 1997), which was used to assess the current psychiatric diagnostic status of participants. Participants were included if their primary diagnosis was generalized SAD. The LSAS is a clinician-administered questionnaire used to assess fear and avoidance symptoms in SAD, through 24 questions on a 4-point Likert-scale (Liebowitz, 1987). A minimum cut-off score of 60 on the LSAS determined inclusion into the SAD groups (Baker et al., 2002; Mennin et al., 2002). The CTQ-SF (Bernstein & Fink, 1998), used to assess EDT, is a self-administered questionnaire that takes approximately 5-10 minutes to complete. A minimum threshold score of 45 on the CTQ-SF was used to determine whether participants had EDT. The CTQ-SF consists of 28 questions, on a 5-point Likert Scale. The CTQ-SF assesses five dimensions of childhood maltreatment: physical abuse, emotional abuse, sexual abuse, physical neglect and emotional neglect (Bernstein et al. 2003).

The neurocognitive test battery comprised tests of verbal fluency, memory, fine motor performance and executive functioning. The pencil and paper tests included tests of verbal memory, executive function and fine motor function.  The verbal memory tests included the Controlled Oral Word Association Test (COWAT), Weschler Memory Scales III (WMS III), and the Hopkins Verbal Learning Test (HVLT). Fine motor function tests completed was the Grooved Pegboard. Executive function testing included the: Stroop Color Word Test (SCWT) and the Trail Making Test (TMT). These tests were followed by two computer tasks of executive functioning: namely the Wisconsin Card Sorting Test (WCST) and the Halstead Category Test (HCT).

*4.3.3 Procedure*

Ethical approval for the study was obtained from the Health Research Ethics Committee at the Faculty of Medicine and Health Sciences, Stellenbosch University. Written, informed consent was obtained from participants. A clinical psychologist assessed participants for the presence or absence of SAD and EDT. Participants underwent neurocognitive testing as part of a larger imaging and genetics study of SAD in the context of EDT.

*4.3.4 Statistical analysis*

Bayesian analysis was the primary statistical analysis used for reporting group differences. SPSS version 19.0 (IBM, 2010) was used to compute NHST descriptive statistics. Bayesian Estimation Supersedes the t-test (BEST) with Markov-Chain Monte-Carlo (MCMC): $\mu_1$ - $\mu_2$ / sqrt($\sigma_1{}^2 + \sigma_2$ ) / 2; statistical analyses were performed to examine the probabilities of difference of the various neurocognitive domains between the groups. An equiprobable prior was chosen for the model assuming initially that both H1 and H0 were true, before statistical computations were run. The MCMC method used was an adaptive Metropolis-within-Gibbs sampler (Roberst & Rosenthal, 2009). BEST estimates the difference in means between two groups and yields a probability distribution over the difference. From this distribution we can take the mean credible value as our best guess of the actual difference with a 95% *Highest Density Interval* (HDI) as the range where the actual credible difference lies. Extreme outliers (age, education level and CTQ) were removed. No group differences were found for demographic variables.

For analysis in BEST, every sample consisted of a burn-in period of 1000 iterations or simulated experiments, based on the standardized model parameters suggested by BEST (Kruschke, 2013). The data sample had a normal distribution. The model was checked for convergence by considering the relevant kernel density plots. The model was also checked diagnostically through the autocorrelation plots within the python BEST program, which showed that throughout the iterative process the autocorrelation was satisfactorily reduced to a nominal amount. In determining the model, uninformative/vague prior parameters were selected, given the relatively novel data and hypotheses being explored. The prior parameters for the model were fixed because of the goodness of fit of data to the model and the fact that the prior parameters were relatively vague. During all runs of the data it was ensured that all of the effect estimates converged to a common figure; this was confirmed by examining all output graphs generated by the BEST. We further used a Bayesian counterpart for correlation

analysis of the various clinical data (CTQ-SF and the LSAS with the neurocognitive tests that were found to be probabilistically different in BEST). We ran a Bayesian counterpart to Pearson correlation in the Python programming language, to simulate MCMC's with a burn in period of 2000 iterations (3 chains) with a total sample of 60 000. The data sample had a normal distribution for each run. The correlation output consisted of odds ratios of the probability of the correlation as the strength/degree of the relationship between variables under investigation. We further defined a region of practical significance (ROPE) (Kruschke, 2012) in order to further estimate whether the probability of the difference was too small to matter. We used a difference of ± 0.1 (Krischke, 2012) on the MCMC - HDI output in order to estimate whether the probability that the true values lie within 0. This would indicate a difference between the groups that would be too marginal to matter. We selected the current ROPE value as a conservative estimate for likelihood of estimating probabilities (Kruschke, 2013), given that we know very little about neurocognitive differences in the aforementioned groups.

## 4.4 *Results*

### 4.4.1 *Descriptive data*

Table 1 presents descriptive statistics for each of the three groups. The mean age of participants was 32.97 years (SD = 8.9). Of the 75 participants 49.3 % were male (n = 37), 73.3% were White (n = 55), 20% mixed race (n=15), 5.3% Black African (n = 4), and one participant was Asian (1.3%). The mean years of education were 15.3 (SD = 2.9). There were no probable statistical differences in mean LSAS score between the SAD groups with and without EDT.

Table 1
Demographic and clinical data

| Characteristic | SAD with EDT | SAD without EDT | Control Participants |
|---|---|---|---|
| Age (y) | 35.5 (SD 9.0) | 33.7 (SD 9.5) | 29.9 (SD 7.6) |
| Gender (Female/Male) (n) | 12/13 | 13/11 | 13/13 |
| Years of Education [a] | 14.7 (2.1) | 15.3 (3.2) | 16.2 (3.2) |
| Ethnicity (n) | Black 2 Colored 8 White 15 | Colored 4 White 20 | Black 2 Colored 3 White 20 Other 1 |
| LSAS Score | 82.6 (58-121) | 80.4 (60-134) | 18.7 (0-41) |
| Fear Score | 41.7 (23-62) | 42.1 (27-63) | 10.4 (0-25) |
| Avoidance Score | 40.9 (29-64) | 38.3 (17-63) | 98.3 (0-20) |
| CTQ Score | 61 (46-89) | 35.5 (26-45) | 31 (25-35) |
| Emotional Neglect | 15.2 (5-23) | 10.0 (5-16) | 7 .1 (5-14) |
| Emotional Abuse | 16.5 (8-25) | 7.4 (5-14) | 6.3 (5-10) |
| Physical Neglect | 9.6 (5-15) | 6.5 (5-13) | 5.6 (5-11) |
| Physical Abuse | 10.6 (5-25) | 6.4 (5-13) | 5.8 (5-13) |
| Sexual Abuse | 8.3(5-19) | 5.2 (5-8) | 5.4(5-12) |
| **MDD** | | | |
| **Current** | 2 (8.0%) | 2 (8.3%) | 0 (0%) |
| **Past** | 12 (48.0%) | 13 (54.2%) | 3 (11.5%) |
| **Neurocognitive Scores** | | | |
| Grooved Pegboard D | | | |
| Grooved Pegboard ND | 65.92 (11.43) | 62.96 (7.46) | 59.62 (5.7) |
| HVLT_Immediate recall | 71.83 (11.26) | 68.59 (10.23) | 65.26 (6.2) |
| HVLT_Delayed recall | 25.60 (4.02) | 25.04 (5.17) | 28.77 (4.5) |
| Stroop Words | 8.44 (2.65) | 8.54 (3.04) | 10.23 (1.7) |
| Stroop Color | 98.40 (20.41) | 108.75 (15.98) | 106.38 (16.11) |
| Stroop ords/Color | 68.00 (12.50) | 74.17 (15.55) | 77.12 (13.87) |
| Trails A | 40.32 (11.40) | 44.92 (10.69) | 48.60 (11.02) |
| Trails B | 28.53 (9.11) | 28.35 (7.28) | 25.02 (7.88) |
| WCST Conceptual level responses | 65.23 (18.29) | 68.90 (24.95) | 57.34 (21.20) |
| WCST Categories completed | 43.88 (10.50 | 44.33 (12.30) | 48.00 (11.24) |
| WCST Non-perseverative errors | 3.40 (1.29) | 3.46 (1.59) | 3.81 (1.47) |
| WCST Learning | 8.36 (3.83) | 8.58 (5.78) | 6.00 (3.58) |
| WCST Perseverative errors | 17.32 (37.77) | 13.08 (34.23) | 10.93 (33.50) |
| WCST Perseverative responses | 7.80 (5.97) | 6.71(3.43) | 7.27 (5.19) |
| WCST Set Fail | 8.88 (8.12) | 7.17 (4.05) | 8.04 (6.05) |
| WCST Total Errors | 0.40 (0.76) | 0.50 (0.89) | 0.38 (0.57) |
| WCST Total Correct | 16.16 (8.02) | 15.29 (8.78) | 13.27 (8.12) |
| CAT Average IL | 47.84 (8.02) | 48.71 (8.78) | 50.73 (8.12) |
| CAT Average Correct | 4.24 (2.83) | 3.67 (1.79) | 3.18 (1.19) |
| CAR Total Time | 39.64 (21.48) | 40.58 (23.62) | 28.69 (15.49) |
| WMS_AL_I | 1909.29 (653.51) | 1740.27 (433.12) | 1604.93 (242.76) |
| WMS_AL_D | 16.32 (3.56) | 14.92 (4.54) | 17.17 (2.39) |
| WMS_LM_I | 8.88 (1.67) | 8.63 (1.66) | 9.38 (0.94) |
| WMS_LM_D | 21.36 (5.77) | 23.79 (5.95) | 26.42 (6.50) |
| | 19.56 (5.82) | 22.92 (6.48) | 24.85 (6.71) |

*Note*. [a] = Years of education starting from primary school through to tertiary education. Definitions: LSAS = Liebowitz Social Anxiety Scale; CTQ = Childhood Trauma Questionnaire; MDD = Major Depressive Disorder (As determined by the Mini International Psychiatric Inventory); HVLT = Hopkins Verbal Learning Test Immediate Recall; WCST= Wisconsin Card Sorting Test CAT = Category Test; CAT WMS AL I = Wechsler Memory Scale Associate Learning Immediate Recall; WMS AL D = Wechsler Memory Scale Associate Learning Delayed Recall; WMS LM I = Wechsler Memory Scale Logical Memory Immediate; WMS LM D = Wechsler Memory Scale Logical Memory Delayed Recall.

### 4.4.2 *Inferential data*

Table 2 provides a summary of the means and HDI for the BEST outputs for each neurocognitive test, for each pairwise group comparison. We discuss the statistical findings for each neurocognitive domain (*Please see* **Appendix A** *for each BEST output graph*).

Table 2

BEST results for neurocognitive tests

| | SAD without EDT and Controls | | | SAD with EDT and Controls | | | SAD with EDT and SAD without EDT | | |
|---|---|---|---|---|---|---|---|---|---|
| | BEST Effect Size | Lower Bound | Upper Bound | BEST Effect Size | Lower Bound | Upper Bound | BEST Effect Size | Lower Bound | Upper Bound |
| GPB Dominant | 0.526 | -0.105 | 1.07 | 0.686* | 0.105 | 1.41 | -0.33 | -1.57 | 0.911 |
| GPB Non Dominant | 0.387 | -0.255 | 0.981 | 0.818* | 0.131 | 1.52 | -0.325 | -1.55 | 0.905 |
| HVLT Immediate | -0.813* | -1.41 | -0.161 | -0.739* | -1.39 | -0.165 | -0.504 | -1.67 | 0.661 |
| HVLT Delayed | -0.651* | -1.24 | -0.0556 | -0.745* | -1.36 | -0.172 | -0.636 | -1.81 | 0.539 |
| Stroop Words | 0.175 | -0.409 | 0.791 | -0.422 | -0.989 | 0.19 | -0.564 | -1.17 | 0.0351 |
| Stroop Colors | -0.298 | -0.843 | 0.346 | -0.716* | -1.28 | -0.0639 | -0.418 | -1.08 | 0.206 |
| Stroop Words/colors | -0.319 | -0.958 | 0.223 | -0.752* | -1.35 | -0.148 | -0.458 | -1.03 | 0.172 |
| Trails A | 0.455 | -0.132 | 1.07 | 0.434 | -0.176 | 1.02 | -0.0534 | -0.599 | 0.582 |
| Trails B | 0.569 | -0.0649 | 1.16 | 0.412 | -0.137 | 1.06 | -0.154 | -0.763 | 0.413 |
| WCST CLR | -0.435 | -1.19 | 0.229 | -0.514 | -1.15 | 0.0987 | -0.208 | -1.01 | 0.517 |
| WCST CATS Completed | 0.337 | -0.918 | 0.317 | 0.657* | 0.0499 | 1.26 | -0.128 | -0.758 | 0.427 |
| WCST NPE | 0.503 | -0.0879 | 1.19 | 0.358 | -0.406 | 1.11 | 0.0684 | -0.554 | 0.705 |
| WCST LRN | 0.268 | -0.395 | 0.968 | -0.251 | -0.443 | 0.11 | 0.11 | -0.594 | 0.776 |
| WCST Perseverative errors | 0.0868 | -0.574 | 0.875 | 0.406 | -0.0744 | 1.43 | 0.214 | -0.629 | 0.997 |
| WCST Perseverative response | -0.055 | -0.66 | 0.689 | 0.0256 | -1.01 | 1.01 | 0.157 | -0.665 | 0.979 |
| WCST Set Fail | 1.07 | -0.465 | 0.455 | 0.618 | -0.24 | 1.56 | -0.381 | -2.088 | 0.988 |
| WCST Total Errors | 0.332 | -0.329 | 1.09 | 0.422 | -0.219 | 1.67 | 0.24 | -0.448 | 1.00 |
| WCST Total Correct | -0.31 | -1.11 | 0.312 | -0.672 | -1.56 | 0.0657 | -0.305 | -1.04 | 0.433 |
| CAT Average IL | 0.498 | -0.141 | 1.3 | 0.455 | -0.219 | 1.00 | 0.12 | -0.467 | 0.803 |
| CAT Average Correct | 0.153 | -0.465 | 0.748 | 0.522 | -0.173 | 1.03 | 0.296 | -0.332 | 0.92 |
| CAR Total Time | 0.324 | -0.232 | 0.98 | 0.543 | -0.0629 | 1.15 | 0.308 | -0.37 | 0.859 |
| WMS_AL_I | -0.617* | -1.2 | -0.0111 | -0.319 | -0.831 | 0.318 | 0.33 | -0.253 | 0.914 |
| WMS_AL_D | 0.172 | -0.985 | 0.513 | 0.000525 | -0.555 | 0.545 | 0.209 | -0.422 | 0.817 |
| WMS_LM_I | -0.394 | -1.00 | 0.159 | -0.827* | -1.41 | -0.228 | -0.465 | -1.02 | 0.179 |
| WMS_LM_D | -0.295 | -0.863 | 0.282 | -0.812* | -1.41 | -0.22 | -0.511 | -1.15 | 0.0465 |

Note. * Statistical difference according to BEST with ROPE > 0.1. Definitions: GPB = Grooved Peg Board; HVLT I = Hopkins Verbal Learning Test Immediate Recall; HVLT = Hopkins Verbal learning Test; Stroop W = Stroop test words; Stroop C = Stroop test colours; Stroop W/C = Stroop Test Words and Colours; WCST CLR = Wisconsin Card Sorting Test conceptual level response; WCST CATS Comp = Wisconsin Card Sorting Test Categories Competed; WCST NPE = Wisconsin Card Sorting Test Non-perseverative errors; WCST LRN = Wisconsin Card Sorting Test learning to learn; WCST PE = Wisconsin Card Sorting Test; CAT = Categories Test; WMS AL I = Wechsler Memory Scale Associate Learning Immediate Recall; WMS AL D = Wechsler Memory Scale Associate Learning Delayed Recall; WMS LM I = Wechsler Memory Scale Logical Memory Immediate; WMS LM D = Wechsler Memory Scale Logical Memory Delayed Recall.

4.4.2.1 *Verbal memory*

Differences in verbal memory were observed between the SAD with EDT and control groups and between the SAD without EDT and control groups. We found a number of verbal memory differences in the SAD with EDT group and control groups. The evidence suggests that the SAD with EDT group performed more poorly on the Hopkins verbal learning immediate recall test (BEST Effect Size -0.739, 95% HDI [-0.165, -1.39]), Hopkins Verbal Learning Delayed Recall Test (BEST Effect Size -0.745, 95% HDI [-0.172, -1.36]); Weschler Memory Scales Logical Memory Delayed Recall Test (BEST Effect Size -0.812, 95% HDI [-0.22, -1.41]) and the Weschler Memory Scales Logical Memory Immediate Recall Test(BEST Effect Size -0.827, 95% HDI [-0.228, -1.41]). The SAD without EDT group performed more poorly on the Hopkins Verbal Learning Immediate Recall Test (BEST Effect Size -0.813, 95% HDI [-0.161, -1.41]), Hopkins Verbal Learning Delayed Recall Test (BEST Effect Size -0.651, 95% HDI [-0.0651, -1.24]); and the Wechsler Memory Scales Logical Memory Immediate Recall (BEST Effect Size -0.617, 95% HDI [-0.0111, -1.2]) compared with controls.

4.4.2.2 *Fine motor functioning*

Fine motor functioning was assessed with the Grooved Pegboard (GPB) test. The SAD with EDT group performed more poorly on the GPB dominant (BEST Effect Size -0.686, 95% HDI [1.41, 0.105]) and non-dominant (BEST Effect Size -0.818, 95% HDI [1.52, 0.131]) hand compared with the control group. There were no differences between the two SAD groups on the GPB. There were no statistical differences observed in fine motor performance in the SAD without EDT group compared with the control group. No correlations were observed between fine motor function and demographic or clinical varables (LSAS and CTQ).

4.4.2.3 *Executive functioning*

There was also evidence for differences in the SAD with EDT group compared with the control group in the Wisconsin Card Sorting Test Conceptual Level Response (BEST Effect Size -0.79, 95% HDI [-1.7, -0.018); and the Wisconsin Card Sorting Test Non-perseverative errors (BEST Effect Size 0.657, 95% HDI [0.0499, 1.26).

4.4.2.4 *Correlations*

Table 3 provides details for correlational tests. Probable relationships were found between the severity of childhood trauma (CTQ-SF total score) and WMS LM immediate recall (Sigma = 9.02, 95% HDI: lower limit = 0.17, upper limit = 1.54, odds ratio = 4.68), HVLT immediate recall (Sigma = 16.58, 95% HDI: lower limit = 0.17, upper limit = 2.80, odds ratio = 16.56), Stroop (Sigma = 12.57, 95% HDI: lower limit = 0.18, upper limit = 2.25, odds ratio = 9.44), GPB dominant hand (Sigma = 15.73, 95% HDI: lower limit = 0.18, upper limit = 2.78, odds ratio = 16.17), GPB non-dominant hand (Sigma = 26.69, 95% HDI: lower limit = 0.17, upper limit = 4.57, odds ratio = 96.67). We also examined whether LSAS scores specifically corresponded with findings in memory tests that were similarly different in each of the SAD groups compared with controls. This analysis included the LSAS score with the WMS immediate recall test, the HVLT immediate recall test and the HVLT delayed recall test. In BEST, these tests were statistically different between each of the SAD groups and the controls. In the SAD with EDT group, there was only a correlation between the LSAS and the HVLT immediate recall test (Sigma = 18.78, 95% HDI: lower limit = 0.16, upper limit = 3.08, odds ratio = 21.70).

Table 3

Bayesian correlations of neurocognitive difficulties in SAD groups

| Group | Clinical | Neurocognitive | Lower (Delta Z) | Upper (Delta Z) | Sigma | odds ratio § |
|---|---|---|---|---|---|---|
| SAD with EDT | CTQ Total | WMS LM I | 1.54 | 0.17 | 9.02* | 4.68 |
| | | WMS LM D | -3.30 | 0.20 | -16.56 | 27.22 |
| | | HVLT I | 2.81 | 0.17 | 16.58* | 16.56 |
| | | HVLT D | -7.20 | 0.21 | -34.81 | 1342.73 |
| | | Stroop C | 2.25 | 0.18 | 12.57* | 9.44 |
| | | Stroop C/W | -1.48 | 0.18 | -7.99 | 4.38 |
| | | WCST NPE | -6.59 | 0.20 | -32.82 | 724.62 |
| | | WCST CLR | -7.94 | 0.19 | -42.35 | 2812.58 |
| | | GPB D | 2.78 | 0.18 | 15.73* | 16.17 |
| | | GPB ND | 4.57 | 0.17 | 26.69* | 96.67 |
| | LSAS Total | HVLT I | 3.08 | 0.16 | 18.78* | 21.70 |
| | | HVLT D | -7.32 | 0.21 | -35.14 | 1504.25 |
| | | WMS LM I | -7.34 | 0.20 | -37.29 | 1535.97 |
| SAD without EDT | LSAS Total | HVLT I | -0.69 | 0.16 | -4.24 | 2.00 |
| | | HVLT D | -10.30 | 0.21 | -49.67 | 29862.18 |
| | | WMS LM I | -0.55 | 0.16 | -3.38 | 1.73 |

*Note.* * Denotes correlations between clinical variable and neurocognitive variable. § The odds ratios represent the degree of likelihood between the correlations; which is the relative probability of the competing hypotheses (1:1), which denotes equiprobable/indistinguishable hypotheses. LSAS Total = Liebowitz social anxiety scale total score; CTQ total = childhood trauma questionnaire total score; WMS LM I = Wechsler memory scales logical memory immediate recall; WMS LM D =Wechsler memory scales logical memory delayed recall; HVLT I = Hopkins verbal learning test immediate recall; HVLT D Hopkins verbal learning test delayed recall; Stroop C = Stroop colour test; Stroop C/W = Stroop words and colours test; WCST NPE = Wisconsin card sorting test non=perseverative errors; WCST CLR = Wisconsin card sorting test conceptual level response; GPB D = grooved pegboard dominant hand test; GPB ND = grooved pegboard non-dominant hand test.

115

*4.5 Discussion*

The present study was conducted to examine the neurocognitive functioning of individuals with SAD and EDT compared to individuals with SAD without EDT and healthy controls. There were a number of noteworthy findings. Firstly EDT appears to play an important contributory role in cognitive fallout in SAD. Secondly the SAD with EDT group demonstrated multiple verbal working memory difficulites, executive functioning difficulites and poorer fine motor performance compared to controls. Thirdly the SAD without EDT group only displayed verbal memory deficits compared to controls. However there were no differences between the two SAD groups in verbal memory.

Verbal memory difficulites were found in both the SAD with EDT and SAD without EDT groups compared with controls. These findings support the available evidence for verbal memory difficulites in SAD. For example, an inability to recall words and auditory verbal episodic experiences in immediate and delayed memory recall trials has been observed in SAD. This points to difficulties involving consolidation and recall in verbal memory processing (Heinrichs & Hofmann, 2004). The only significant difference between the SAD without EDT group and controls was in the HVLT immediate recall task. The HVLT generally measures verbal memory with regard to verbal working memory, basic verbal learning capacity, verbal recognition and verbal memory recall (Lacritz & Cullum, 1998). Our findings in the SAD without EDT group are indicative of cognitive performance difficulites on verbal retrieval on immediate recall and verbal working memory but not verbal learning. Individuals with anxiety disorders display less residual verbal working memory capacity (Leigh & Hirsch, 2011). Leigh and Hirsch (2011) found that individuals whose cognitive worry content is verbal rather than imagery-based, deplete working memory capacity. High levels of anxiety and worry have also been associated with reduced working memory capacity (that is, a limited capacity store needed to engage in different tasks and switch between tasks) (Hayes & Hirsch, 2007). Verbal processing in working memory of social fears is, therefore, an important component of SAD (Nilsson, et al 2012). Disordered verbal memory may be a mechanism involved in the maintenance of the disorder.

EDT compromises a number of vulnerable brain regions -the hypothalamic–pituitary–adrenal (HPA) axis, amygdala, hippocampus, and prefrontal cortex - which are linked to dysfunctional neurocognitive process throughout adult life (Wilson et al, 2011). The SAD with EDT group displayed memory impairment on both the HVLT and WMS tests. However,

116

there was no difference in the degree of impairment between the SAD groups. The SAD with EDT group displayed impairment in delayed verbal memory processing in addition to immediate recall, which is indicative of reduced or impaired verbal learning and long term verbal memory. Verbal learning and long-term memory has not been fully investigated in SAD, but has been a finding in some studies of PTSD. Traumatic stress has been suggested to effect the production of long-term memory by producing effects on memory consolidation through basolateral amygdala activation (Chavez et al, 2013) and stress pathways of the HPA axis (Finsterwald & Alberini, 2014). Chronic stress mostly impairs hippocampal-dependent mechanisms and functions and not amygdala related memory processing, which may, in part, explain long-term memory dependent mechanisms (Finsterwald & Alberini, 2014). Stressors and traumas impair cognitive tasks and related memory functions that are more complex (including verbal memory processing) but may improve simpler cognitive tasks, such as spatial memory processing (Finsterwald & Alberini, 2014). In particular, severe or chronic stress has a negative effect on hippocampal morphology and function, likely contributing to the negative effect of stress on hippocampal-dependent memories (Conrad, et al., 1999, Kirschbaum et al., 1996). EDT may be a mediating mechanism in learning and long-term memory, where verbal long-term memory impairment is observed.  Exposure to severe acute or chronic stress, including EDT, over the developmental life span, can have a negative impact on the neuronal morphology in the hippocampus (Finsterwald & Alberini, 2014).

Our memory findings need to be carefully examined. Heinrichs and Hofmann (2004) found that memory encoding in high socially anxious individuals for threat information was poorer than individuals who were low on social anxiety. In their study they examined a release from a proactive interference technique to explore memory encoding in SAD. The assumed hypothesis was that memory encoding for social threat would be higher in individuals with high social anxiety rather than low social anxiety (Heinrichs & Hofmann, 2004). Most studies that have been conducted appear to focus predominantly on attention and memory processing (Amir & Bomyea, 2010). These specific areas of deficit are also supported by numerous neuroimaging studies that have indicated that areas of the brain, such as the temporal lobes, hippocampi and amygdala, may be dysregulated in individuals with SAD (Airaksinen, 2004). It has also been speculated that individuals with SAD are likely to have more threat related biased episodic memory in social experiences (Wild et al., 2008). A study by Pérez-López and Woody (2001) showed that individuals with SAD had less accuracy in recognizing previously seen photographs of individuals than controls when experiencing increased state

117

anxiety (Pe ́rez-Lo ́pez & Woody, 2001). Some studies has found no significant impairments in memory processing in individuals with SAD (Lundh & Ost, 1997; Rapee, McCallum, Melville, Ravenscroft & Rodney, 1994). Coles and Heimberg (2005) found no significant memory biases, specifically for threat relevant information in SAD compared with other anxiety disorders (panic disorder, posttraumatic stress disorder and obsessive compulsive disorder).   Interestingly, we found no correlations between verbal memory tests and CTQ-SF scores. EDT may play a contributory role in the neurocognition of memory in SAD, by providing information of particular pathophysiological mechanism, such as the possible greater recruitment of hippocampal processing or dysfunction. This may be more clearly observed through the seminal observation of Yerkes and Dodson, in which various studies have observed a nonlinear relationship between stress intensity and cognitive performance in rodent models and humans. For example, variation of the intensity of a stress intrinsic to a learning paradigm, such as water temperature in a radial arm water maze, demonstrated the inverted U effect of stress on learning and memory performance in rats (Salehi, Cordero, & Sandi, 2010; Finsterwald & Alberini, 2014). It can be assumed that EDT may contribute to the pathophysiology by affecting other neurocognitive functions, as the SAD with EDT group displayed difficulites in other neurocognitive domains, such as executive functioning. This may be important, as executive functioning problems have been found in individuals who have experienced EDT.

Few studies that have examined executive functioning in SAD. The low executive functioning in patients with SAD is consistent with previous studies (Fujii et al, 2013, Asmundson et al., 1994; Cohen et al, 1996; Graver & White, 2007; Topcuoglu, et al., 2009). However, none of these studies examined the effects of EDT. We found differences on two executive functioning tests in the SAD with EDT group compared with controls: the Stroop test and the WCST. The WCST assesses a wide range of cognitive processes related to executive functioning (Lezak et al, 2012). Fujii et al. (2013) examined 30 SAD participants and 30 controls matched on gender, education level, and age, and found that the SAD group performed more poorly on the WCST than controls. In the present study, we found differences between the SAD with EDT group and controls. Our findings closely match Fujii et al (2013); however, the SAD without EDT group showed no significant differences in executive functioning compared to the SAD with EDT group. The SAD with EDT group performed more poorly compared to controls on the WCST in the areas of conceptual level responses and errors (non-perseverative), suggesting that EDT may play a mediating role in

executive functioning difficulites in SAD. Recent research has demonstrated that exposure to stress is sufficient to cause significant structural remodeling of the principle projection neurons within the rodent prefrontal cortex (de Kloet et al., 2005; Holmes & Wellman, 2009; Sapolsky, 2003). In parallel, there is growing evidence that stress induced alterations in prefrontal cortex neuronal morphology are associated with deficits in rodent executive functions such as working memory, attentional set-shifting and cognitive flexibility, as well as emotional dysregulation in the form of impaired fear extinction (Holmes & Wellman, 2009).  The prefrontal cortex is an important area that is affected by EDT; it is also the location of executive functions. (Wilson et al, 2011). Illustrating this, Beers and DeBellis (2002) found that children with maltreatment-related PTSD performed more poorly on measures of attention and executive functioning, for example, on the Stroop Color and Word Test, Digit Vigilance Test, WCST, and COWAT. The executive functioning difficulites in the SAD with EDT group on the WCST portray a particular aspect of executive functioning error, which appears to be predominantly in shifting set. This is in accordance with the sparse evidence indicating that anxiety is associated with reduced attentional control and working memory difficulites, related to shifting set (Derryberry & Reed, 2002; Eysenck, 1979; Eysenck & Calvo, 1992). In conclusion, we infer that poorer executive functioning characterises SAD in combination with EDT.

Fine motor functioning has not been fully investigated in SAD and no published studies have explored fine motor functioning in individuals with SAD. One of the earliest studies of anxiety on fine motor performance conducted by Terelak (1990), examined 145 adolescent males and found that anxiety had a negative impact on motor performance and eye-hand coordinated fine motor learning. The SAD with EDT group demonstrated slower fine motor response speed compared with the control group. Similar findings of psychomotor slowing have been observed in OCD studies (Basso et al., 2001); this was demonstrated in similar tasks of fine motor performance and in tasks of response inhibition where individuals with OCD had difficulties modulating neurobehavioral inhibition. Fine motor difficulties have not been extensively studied in anxiety disorders; however, in a recent study, Skirbekk et al. (2012) found significant motor impairment in children with anxiety disorders compared with healthy controls. Performance difficulties in motor functioning may therefore be attributable to EDT in combination with SAD.

In summary our findings suggest that when SAD occurs within the context of EDT there is a likelihood of more neurocognitive difficulites. It appears that EDT may be a contributory factor in the aetiopathophysiology of SAD for the development of neurocognitive dysfunction. Our findings may, in part, explain the lack of neurocognitive findings in many of the SAD studies that have examined neurocognition, however a closer examination of aetiological variables is warranted (Sutterby & Bedwell, 2012; O'Toole & Pederson, 2011).

*4.6 Conclusion*

These findings support memory difficulties in verbal memory specifically in SAD  The SAD with EDT group demonstrated a significant number of neurocognitive difficulties in addition to memory difficulties, which included executive and fine motor functioning. No differences were found between the SAD groups on any neurocognitive domain making it difficult to establish precise effects of EDT on the neurocognitive mechansims in SAD (*See* **Appendix B** *for illustrative bar chart on neurocognitive differences between the two SAD groups*). A number of neurocognitive domains were not tested in our study (for example: visual-spatial functioning and visual memory) and we recommend examination of those domains in SAD with EDT. An important limitation worth mentioning in this study was the use of self-reported retrospective measure of childhood trauma (CTQ) and findings of impaired memory processing in the SAD with EDT group. Memory processing difficulties may relate to poor self-reporting of historical events such as childhood trauma. The use of a Bayesian statistical approach is a strength of this study. It allowed for generation of a set of posteriors that may be further tested in future research. Bayesian testing also allowed us to examine a large range of neurocognitive tests in a limited sample without being constrained by NHST statistical parameters of evaluating a large number of data points in relatively smaller samples (Gelman et al., 2012).

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# 5. STRUCTURAL MAGNETIC RESONANCE IMAGING (SMRI) IN SOCIAL ANXIETY DISORDER IN THE CONTEXT OF EARLY DEVELOPMENTAL TRAUMA: A BAYESIAN ANALYSIS

**Chapter 5 presents the sMRI findings. They have been presented here in the format of a publication, as this section is being prepared for publication.**

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5.1 Abstract

Background: There are no studies to date that have examined structural and volumetric (morphometric) abnormalities of the brain of individuals with social anxiety disorder (SAD) with early developmental trauma (EDT). The current study used Free Surfer to examine differences in brain volumes between participants (n = 70) with SAD and EDT (n = 23), SAD without EDT (n = 22) and healthy controls (n = 25). The mean age of participants was 33.19 years (SD = 9.9)

Methods: Participants were screened with the Mini International Neuropsychiatric Interview (MINI), Liebowitz Social Anxiety Scale, (LSAS) and Childhood Trauma Questionnaire (CTQ). Scanning was carried out on a 3T Siemens Allegra scanner, scanning entailed structural magnetic resonance imaging (sMRI) of the limbic system circuitry and associated neurocircuitry. Freesurfer imaging software was used to post-process the sMRI images. We used Bayesian statistical analyses to compare group differences.

Results: The left and right anterior cingulate cortex and the left thalamus were smaller in the SAD with EDT compared with controls and the left caudate nucleus was smaller in the the SAD with EDT compared with the SAD without EDT group. There were no differences between the SAD without EDT group and controls.

Discussion: Our findings suggest greater specificity of the ACC in the pathophysiology of SAD with EDT. Individuals with SAD alone do not appear to display significant reductions in grey matter volumes compared with controls, however EDT appears to play a specific role in grey matter volume reductions in the ACC and left thalamus, in individuals with SAD who have experienced EDT.

*5.2 Introduction*

Social anxiety disorder (SAD) is classified as an anxiety disorder in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5, APA 2013). The underlying neuroanatomical pathophysiological processes of SAD are poorly understood and understanding these processes may help clinicians better diagnose, treat and possibly prevent SAD (Talati et al, 2013). To date there are few studies that have examined the neuroanatomical abnormalities in individuals with SAD (Pannekoek, et al, 2013; Talati et al, 2013). The few structural magnetic resonance imaging (sMRI) studies that have been conducted have used varying approaches and have reported inconsistent results (Pannekoek, et al, 2013). Some studies have demonstrated no volumetric abnormalities and other studies have demonstrated a decrease in the volume of the amygdala, the posterior right temporal gyrus and the hippocampus (Irle et al., 2010; Liao et al., 2011; Potts et al., 1994, Pannekoek, et al., 2013).

There are currently no sMRI studies that have examined structural or morphological brain abnormalities in SAD within the context of EDT. There is some compelling evidence that SAD has strong childhood developmental origins (Ollendick, Hirshfield & Becker, 2002). Early developmental traumas (EDTs) constitute a significant risk in the development of SAD during childhood and in later adult life (Kuo et al, 2011; Erwin et al., 2006). Disruption in social-emotional-developmental neurocircuitry may result, among other factors, from dysfunctional parental modeling or EDT. EDT is broadly defined as any traumatic experience that occurs before the age of 18 years (Bernstein, 1998). On average, one in ten children annually have been neglected or psychologically abused. This approximates to between 4-16% of children having been abused (Gilbert et al, 2008). These figures represent findings from American samples, so this may underestimate the prevalence of abuse in children in South Africa and other developing countries.

There are a number of brain circuits involved in the pathophysiology of SAD. The limbic system has been considered to be an important area of focus with regard to SAD. It is a brain region comprising interconnected structures involved with emotion, motivation and memory (Detweiler, Comer & Albano, 2010). A particular anatomical structure in the limbic system, the amygdala, plays a significant role in fear learning, and social fears in particular, in SAD (Birbaumer et al., 1998; Etkin & Wager, 2007; Rosen & Schulkin, 1998; Shin & Liberzon, 2010; Detweiler, Comer & Albano, 2010). There have been a number of neuroimaging

studies focusing predominantly on brain functioning in the limbic system and amygdala in SAD; with functional magnetic resonance imaging (fMRI) being one of the main investigative modalities (Ferrari et al, 2008; Brühl et al, 2014). Although fMRI studies have focused predominantly on understanding functional and physiological brain processes underlying SAD, they may be limited as the process of scanning may elicit performance anxiety, which in itself may bias fMRI study data in SAD studies (Talati et al, 2013). The benefits of conducting an sMRI study is that sMRI is largely state-independent and may provide information about structural processes involved the pathophysiology of SAD (Talati et al, 2013). At present there are few sMRI studies of SAD. In a 2008 (Ferrari et al, 2008) review of the neuroimaging literature of SAD, only one sMRI study was found. To date, there have been only two sMRI studies of SAD (Potts et al, 1994; Talati et al,2013).

In a review of neuroimaging studies of SAD, Freitas-Ferrari et al. (2010) found the amygdala and its connections (in the emotional and fear circuitry) to play a key role in SAD (Freitas-Ferrari et al., 2010). The insula (Shah et al., 2009), anterior cingulate cortex (ACC) (Amir et al., 2005; Blair et al., 2011) and ventromedial prefrontal cortex have also been reported to be involved in SAD (Damsa et al., 2009; Etkin and Wager, 2007; Freitas-Ferrari et al., 2010, Pannekoek, et al., 2013). Additionally, dysfunction of corticolimbic circuitry, limbic, paralimbic and disinhibition of the medial prefrontal cortex (MPFC) are hypothesized to be associated in the pathophysiology of SAD (Ding, Chen, Qiu, Liao, Warwick, Duan, Zhang & Gong, 2011; Liao, Xua, Mantinib, Dinga, Machado-de-Sousac, Hallakc, Trzesniakc, Qiud, Zenga, Zhangd, Crippac, Gonge, Chen, 2011). Few studies have adequately examined cortico-striatal pathology and associated grey matter abnormalities SAD (Sareen et al., 2007; Freitas-Ferrari et al. 2010). Further, the few morphological studies of drug-naïve SAD patients have shown no significant difference in respect to total cerebral, caudate, putamen, and thalamic volumes compared with controls (Potts et al., 1994). More recently a number of grey matter (GM) abnormalities have been identified in SAD (Potts et al, 1994; Talati, Pantazatos, Schneier, Weissman & Hirsch, 2013). Compared with controls these ranged from increased GM in the left parahippocampal, middle occipital, bilateral supramarginal and angular cortices and the left cerebellum; and lower GM in the bilateral temporal poles and left lateral orbitofrontal cortex (Talati et al., 2013). Cerebellar, parahippocampal, and temporal pole differences have also been observed in SAD compared with controls (Talati,et al., 2013).  Lower gray matter (GM) volumes of the amygdala, hippocampus (Irle, 2010), bilateral temporal poles and left lateral orbitofrontal cortex (Talati et al., 2013); greater GM

in the left middle occipital, bilateral supramarginal, angular cortices, and the left cerebellum (Talati et al., 2013); and bilateral cortical thinning in fusiform and post-central regions (Syal et al., 2012) have been found in SAD relative to controls.

Talati et al. (2013), in comparative study of GM differences in SAD patients, found greater GM in the parahippocampal and middle occipital, and bilateral supramarginal and angular cortices, and left cerebellum; and lower GM in bilateral temporal poles and left lateral orbitofrontal cortex compared with controls. In a study Cassimjee et al (2010) who examined regional brain volume changes in 11 prticipants with SAD treated with an antidepressant medication, escitalopram, they found decreases in brain volume in the bilateral superior temporal cortex, vermis and the left cerebellum following, 12-weeks of treatment.

In a resting state fMRI study by Pannekoek et al (2013), individuals with SAD demonstrated increased negative right amygdala connectivity in the left middle temporal gyrus compared with controls. They also showed an increase in the left supramarginal gyrus and left lateral occipital cortex. With regards to the salience network, individuals with SAD have increased positive bilateral dorsal anterior cingulate connectivity with the left precuneus and left lateral occipital cortex compared with controls (Pannekoek, et al, 2013). Dannlowski et al (2012), in a study of traumatic stress in early life, found a strong association between EDT and amygdala responsiveness to threat-related facial expressions. Their sMRI analysis yielded reduced gray matter volumes in the hippocampus, insula, orbitofrontal cortex, anterior cingulate gyrus, and caudate, in subjects with increased levels of EDT. The associations did not appear to be influenced by trait anxiety, depression level, age, intelligence, education, or a more recent stressful life event (Dannlowski et al, 2012).

Given the absence of well-established studies detailing structural abnormalities for SAD within the context of EDT, we compared participants with DSM-IV generalized SAD with EDT, SAD without EDT and healthy controls to identify GM differences between the two groups (i) to identify brain abnormalities associated with SAD with EDT compared with SAD without EDT and controls; (ii) to examine specificity of these findings of SAD with EDT compared to SAD without EDT.

## 5.3 *Methods*

### 5.3.1 *Participants*

70 participants were included: 22 with SAD with early developmental trauma, 23 subjects with SAD without early developmental trauma and 25 age, gender, ethnicity, language, handedness and education matched control participants. Participants were all older than 21 years and were fluent in English.

### 5.3.2 *Procedure*

Ethics approval for human research was obtained through the Health Research Ethics Committee at the Faculty of the Health Sciences, Stellenbosch University. Written, informed consent was obtained from all participants before proceeding with data collection. A trained clinician conducted diagnostic interviews with participants for the presence or absence of SAD and EDT. Following the diagnostic assessment participants attended a follow up visit within two weeks for sMRI scanning. The study was part of a larger study of the mechanisms of SAD within the context of EDT, which examined imaging, genetics and neurocognitive findings.

### 5.3.3 *Instruments*

Subjects were screened with the Mini International Neuropsychiatric Interview (MINI) version 6.0 (Sheehan et al., 1998) to determine whether they met inclusion criteria for one of the three groups. The Liebowitz Social Anxiety Scale (LSAS) was used to determine SAD symptom severity in the SAD participant groups (Heimberg et al. 1999). The LSAS is a 48-item clinician rated scale that assesses the level of anxiety (24 items) and avoidance (24 items) in a broad range of social and performance situations (Leibowitz 1987). EDT was assessed using the childhood trauma questionnaire – short form (CTQ – SF). The CTQ-SF is a 28 item self report measure of childhood trauma. The CTQ-SF measures five domains of childhood trauma: emotional abuse, emotional neglect, physical abuse, physical neglect and sexual abuse (Bernstein, 1998).

### 5.3.4 *Demographics*

Both right-handed and left-handed participants were included and carefully matched across groups. Exclusion criteria comprised: all DSM-IV psychotic disorders, bipolar mood disorder, obsessive-compulsive disorder, eating disorders and current alcohol or substance abuse or addiction disorders. Other Axis one disorders where only included if they were in

remission or they were not primary diagnoses, as determined with the MINI. All neurological disorders, including head injuries with a loss of consciousness were also exclusions. Other criteria for exclusion were drug abuse/dependence or alcohol abuse/dependence within the past 6 months (Cooney et al., 2006). Participants on current psychotropic medication (with the exception of an SSRI) were excluded. Participants on SSRIs were asked not to take their medication on the day prior to their MRI scan. With regards to the MRI, participants were excluded if they had a cardiac pacemaker, metal prosthesis or pin(s), clips on blood vessels, inner ear prosthesis, an infusion pump, a metal intra-uterine contraceptive device or they were currently pregnant. All metal objects and jewellery were removed before any scanning took place.

### 5.3.5 *Data acquisition and analysis*

### 5.3.5.1 *MRI parameters*

Each scanning session lasted approximately one hour.  Conventional structural data was acquired.  A Siemens 3 Tesla Allegra fMRI scanner at the Cape Universities Brain Imaging Centre (CUBIC at Tygerberg Campus) was used to acquire images. A high resolution T1-weighted 3D MPRAGE structural image was first obtained with the following parameters: sagittal orientation, TR =2300ms, TE = 3.93ms, FOV = 220mm, 160 slices; 256x256 matrix; 1x1x1mm^3 resolution; 9 minute scan time.

In order to acquire region specific brain volume measurements, we carried out further analysis using Freesurfer, a brain imaging software package designed for the study of cortical and subcortical anatomy (Fischl & Dale, 2000). Freesurfer's processing stream consists of several different stages: volume registration with the Talairach atlas, bias field correction, initial volumetric labeling, non-linear alignment to the Talairach space and a final labeling of the volume (Dale & Fischl, 1999). One of the major benefits of Freesurfer is its ability to carry out all processing steps automatically, however its processing pipeline is computationally intensive; we, therefore, utilized custom batching scripts on the Centre for High Performance Computing (CHPC) in Rosebank, Cape Town (http://www.chpc.ac.za).

### 5.3.5.2 *Data analysis*

Intracranial volume (ICV) was accounted for in the statistical analysis in Bayesian testing by factoring differences in ICV in the analysis. Locations were indicated on sample MRI images using ROI selections from the Mango (**M**ulti-Image **An**alysis **G**UI) imaging software

application (http://rii.uthscsa.edu/mango). The following grey matter structures were examined: putamen, thalami, hippocampi, caudate nucleus, ACC, amygdala, and frontal cortex (see Table 5 for more information on means).

A Bayesian analysis of probabilities of group differences was used, rather than null-hypothesis significan testing (NHST) to examine neuroanatomical structural differences across the groups. In NHST the p value generated may be misleading, in that it fails to provide the information that a researcher actually wants to have (Masson, 2011). The p value is a conditional probability that indicates the likelihood of an observed result given that the null hypothesis is correct. Using a Bayesian approach to our data analysis allowed us to consider probability rather than significance, thereby avoiding type I errors in hypothesis testing (Gelman, et al., 2012).

We used Bayesian estimation supersedes the t-test (BEST) with Markov-Chain Monte-Carlo; the MCMC method used is an adaptive Metropolis-within-Gibbs sampler (Roberts & Rosenthal, 2009). BEST estimates the difference in means between two groups and yields a probability distribution over the difference. A mean credible value was taken as an estimate of actual difference and a 95% *Highest Density Interval* (HDI) was taken as the range where the actual difference had 95% credibility. Best was calculated in the Python programming language using Kruschke's BEST protocol and code (Kruscke, 2013; Kruschke, 2011). For the analyses in BEST, every sample consisted a burn-in period of 1000 iterations or simulated experiments, based on the standardized model parameters suggested by BEST (Kruschke, 2013). The Bayesian data analysis examined the probabilities of the likelihood of our data findings (posteriors) given the evidence (priors). Our priors were uninformative, as there is no informative prior knowledge about the specific hypotheses under examination (there is no Bayesian information on sMRI and data on SAD within the context of EDT). The data sample had a normal distribution. The model was checked for convergence by considering the relevant kernel density plots and was also checked diagnostically through the autocorrelation plots within the python BEST program; which showed that throughout the iterative process the autocorrelation was satisfactorily reduced to a nominal amount. During all runs of the data it was made sure that all of the effect estimates converged to a common figure; this was confirmed by examining all output graphs generated by the BEST.

## 5.4 *Results*

### 5.4.1 *Descriptive data*

Table 4 presents descriptive statistics for each of the three groups. The mean age of participants was 33.19 years (SD = 9.9). Of the 70 participants, 47.1 % were male (n = 33) and 52.9 % female (n = 52.9). 76.1% were White (n = 54), 15.5% Coloured (n=11), 5.6% Black African (n = 4), and one participant was Asian (1.4%). The mean years of education were 15.4 (SD = 3.3). There were no statistically probable differences in mean LSAS score between the SAD groups with and without EDT *(BEST Effect Size 0.0830, 95% HDI [-0.522, 0.680]).*

Table 4

Sample characteristics in sMRI study

| Characteristic | SAD with EDT | SAD without EDT | Control Participants |
|---|---|---|---|
| **Age (y)** | 37(20-62) | 33 (21-59) | 30 (22-47) |
| **Gender (Female/Male) (n)** | 9/13 | 11/12 | 13/12 |
| **Years of Education** [a] | 14 (10-19) | 16 (12-23) | 15 (11-19) |
| **Ethnicity (n)** | Black 1<br>Colored 6<br>White 14<br>Other 1 | Colored 2<br>White 21<br>Other 1 | Black 3<br>Colored 3<br>White 19 |
| **LSAS Score** | 80 (-121) | 78 (60-134) | 20 (0-53) |
| **Fear Score** | 41 (25-60) | 43 (27-71) | 11 (0-26) |
| **Avoidance Score** | 39 (19-64) | 36 (17-63) | 10 (0-27) |
| **CTQ Score** | 63 (44-92) | 35 (26-39) | 31 (25-39) |
| **Emotional Neglect** | 16 (5-24) | 10 (5-16) | 7 (5-14) |
| **Emotional Abuse** | 17 (8-24) | 7 (5-13) | 7 (5-10) |
| **Physical Neglect** | 9 (5-21) | 6 (5-13) | 6 (5-11) |
| **Physical Abuse** | 11 (5-25) | 7 (5-13) | 6 (5-13) |
| **Sexual Abuse** | 9 (5-25) | 5 (5-8) | 5 (5-12) |

*Note*. Abbreviations: [a] Years of education starting from primary school through to tertiary education; LSAS: Liebowitz Social Anxiety Scale; CTQ: Childhood Trauma Questionnaire. *Note*. There were no differences between the SAD groups in the LSAS (BEST effect size = 1.83, 95% CI [-11.7, 15.2]) and there was a difference between the CTQ between the SAD with EDT and the SAD without EDT (BEST effect size = 28.7, 95% CI [20.6, 35.6) and control (BEST effect size = 31.4 CI [23.7, 38.7]) groups.

Table 5

sMRI means and standard deviations for each group

| Grey matter structure | Controls Mean | Controls SD | SAD with EDT Mean | SAD with EDT SD | SAD without EDT Mean | SAD without EDT SD |
|---|---|---|---|---|---|---|
| Left Hippocampus | 3964.24 | 465.51 | 3904.00 | 367.22 | 3885.18 | 475.79 |
| Right Hippocampus | 4003.04 | 448.50 | 3989.09 | 429.72 | 4192.68 | 642.18 |
| Left Caudate | 3806.88 | 755.32 | 3823.57 | 1017.96 | 3606.68 | 349.05 |
| Right Caudate | 3995.76 | 728.89 | 3975.96 | 444.99 | 3754.00 | 383.34 |
| Left Putamen | 6065.64 | 1021.36 | 6357.22 | 668.01 | 6119.09 | 825.46 |
| Right Putamen | 5804.68 | 996.63 | 6064.57 | 793.59 | 5858.82 | 724.02 |
| Left Amygdala | 1612.24 | 279.99 | 1633.83 | 301.30 | 1693.09 | 190.57 |
| Right Amygdala | 1679.08 | 274.19 | 1738.96 | 231.51 | 1740.00 | 193.15 |
| Left Thalamus | 6878.92 | 853.42 | 6841.78 | 734.18 | 6523.50 | 602.04 |
| Right Thalamus | 7148.00 | 789.32 | 7142.70 | 702.71 | 6840.45 | 641.87 |
| Left Nucleus Accumbens | 798.52 | 131.07 | 809.39 | 124.10 | 791.55 | 158.24 |
| Right Nucleus Accumbens | 806.32 | 154.09 | 819.52 | 133.16 | 749.18 | 131.50 |
| Left ACC | 2010.72 | 512.74 | 1871.78 | 576.17 | 1854.55 | 348.64 |
| Right ACC | 2077.24 | 507.83 | 2115.30 | 427.94 | 2322.91 | 430.23 |
| Left Frontal | 818.00 | 140.33 | 839.87 | 179.09 | 815.86 | 156.74 |
| Right Frotnal | 1104.64 | 245.24 | 1076.17 | 141.36 | 1007.86 | 207.55 |

*Note.* Each table represents standard deviations and modes unadjusted by ICV, for each of the pertinent grey matter limbic system volumes under investigation in this study.

### 5.4.2 Inferential data

### 5.4.2.1 sMRI Data

Volume data from Freesurfer was computed through our software pipeline, with pairwise testing conducted using BEST. The purpose of the analysis was to estimate probabilities of difference between the three groups. BEST outputs provided evidence for grey matter volume differences in individuals with SAD without EDT compared to controls and in individuals with SAD with EDT compared to controls. Both SAD groups were also compared with each other. The BEST analysis (see Figure 6) found a number of probable volume differences in the SAD with EDT group compared with the control group and with the SAD without EDT group. These findings indicate unique structural differences between SAD with EDT and SAD without EDT. There have been mixed findings with regards to structural volumetric abnormalities in SAD, with some studies finding brain volumetric abnormalities and others finding none. We found no probability of difference between the groups (SAD with EDT and SAD without EDT) in the hippocampi, frontal lobe, right caudate nucleus, right thalamus and the amygdala. The SAD without EDT group in our study did not show any structural differences compared to the control group. Probable differences were found in the left caudate nucleus between the SAD with EDT and SAD without EDT groups and the left thalamus and the ACC in the SAD compared with controls.

Each of the structural locations that exhibited probable statistical volume differences will now be discussed (with statistical findings):
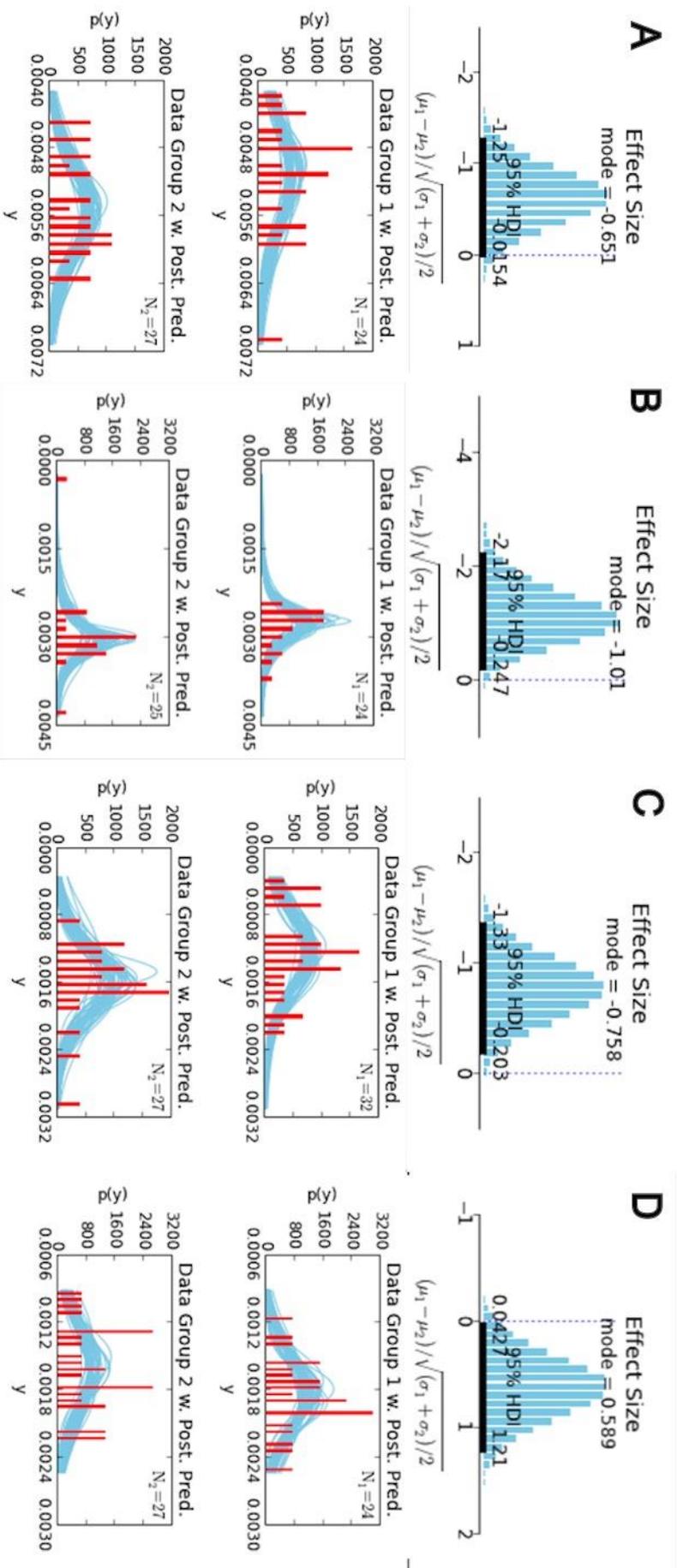
Figure 6 BEST and MCMC of brain volume differences: **(A)** Left Thalamus in SAD with EDT group showing effect size indicating probable difference in distribution; **(B)** BEST of Left Caudate Nucleus in SAD with EDT group and SAD without EDT group showing effect size indicating probable difference in distribution; **(C)** BEST of Left ACC in SAD with EDT group showing effect size indicating probable difference in distribution; **(D)** BEST of Right ACC in SAD with EDT group showing effect size indicating probable difference in distribution.

143

*(i) Left Thalamus*

Bayesian statistical analysis detected a substantial probable difference in volume in the Left Thalamus of the SAD with EDT group compared with controls *(BEST Effect Size -0.651, 95% HDI [-0.0154, -1.25])*, as seen in Figure 7 of the BEST output. The left thalamus was substantially smaller in the SAD with EDT group compared with the control group.

*(ii) Left Caudate Nucleus*

Bayesian statistical analysis detected a substantial probable difference in volume in the left caudate nucleus between the SAD with EDT and SAD without EDT groups *(BEST Effect Size -0.651, 95% HDI [-0.0154, -1.25])*, as seen in Figure 8 of the BEST output. The volume of the left caudate was smaller in the SAD with EDT group compared with the SAD without EDT group. There was no difference in the caudate volume between either SAD group and the control group. This may indicate a difference in pathophysiology between individuals with SAD with EDT.

*(iii) Right and Left Anterior Cingulate Cortex (ACC)*

The BEST analysis found a substantial difference in volume in the left ACC *(BEST Effect Size -0.758, 95% HDI [-0.203, -1.33]), and the right ACC (BEST Effect Size 0.589, 95% HDI [1.21, 0.0427])* in the SAD with EDT group compared with the control group (refer to Figure 9).

144
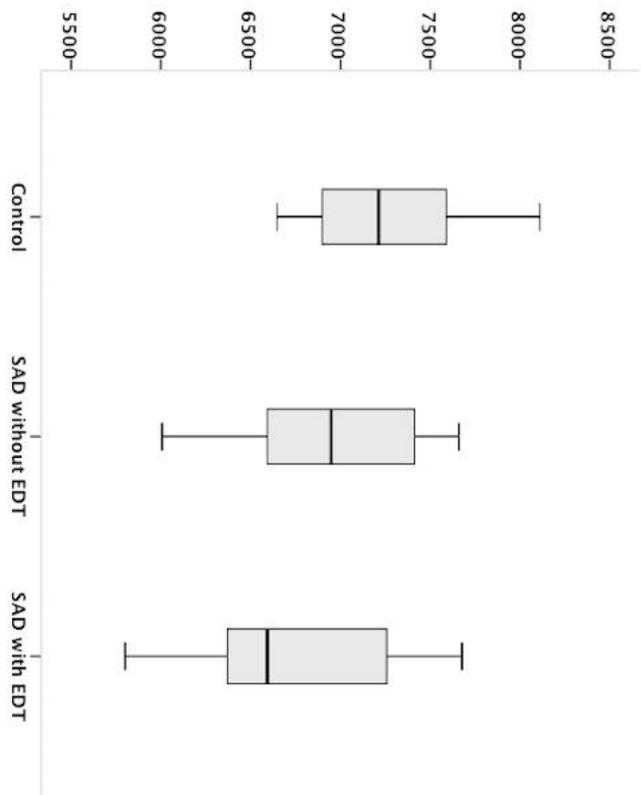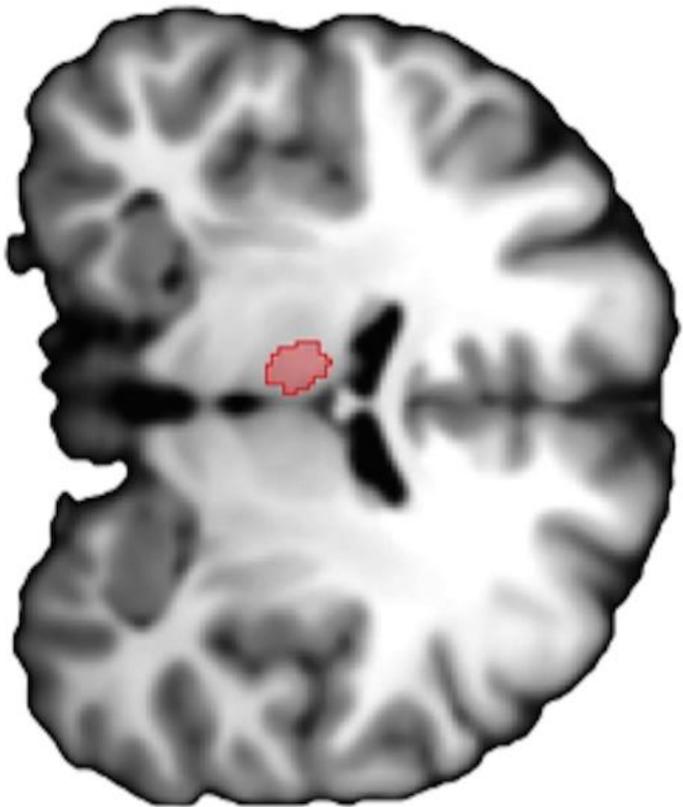
Figure 7 There was statistical difference in the left thalamus between the SAD with EDT group and the control group (M = -0.65 95% HDI [-0.02, -1.25]).
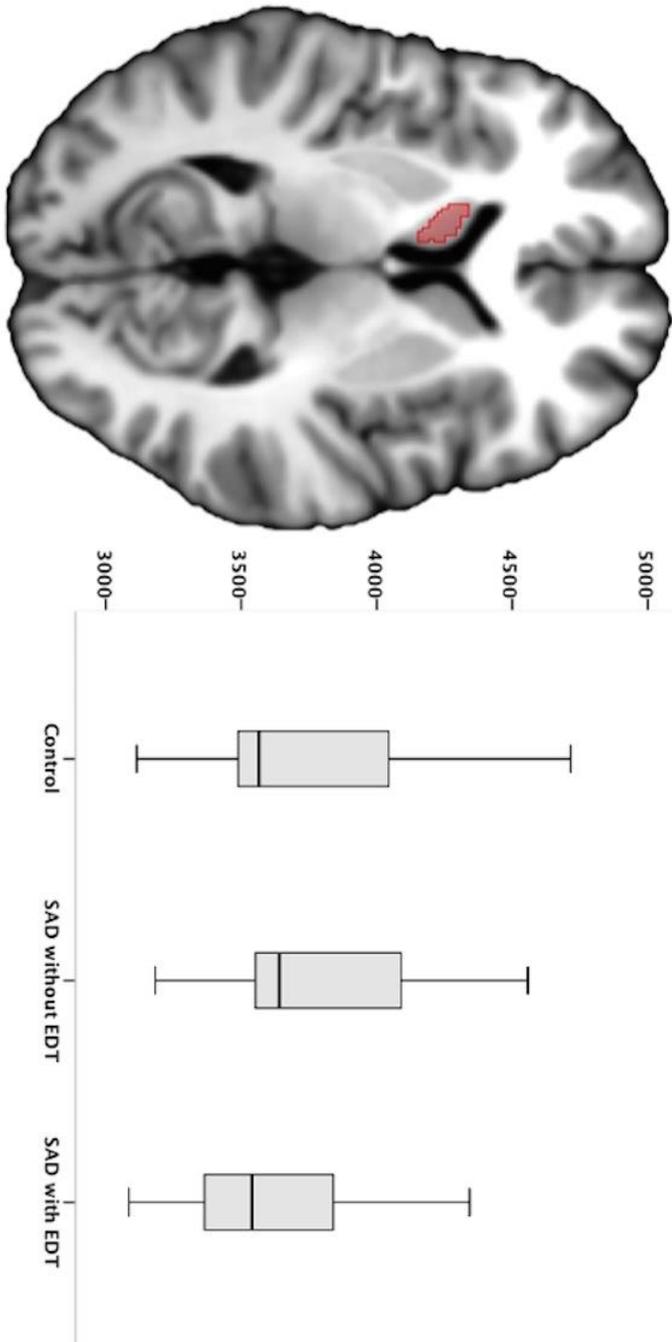
Figure 8 There was statistical difference in the left caudate nucleus between the SAD with EDT group and the SAD without EDT group (M = -1.01. 95% HDI [-0.25, -2.17]). There were no significant differences in the SAD without EDT group.

146
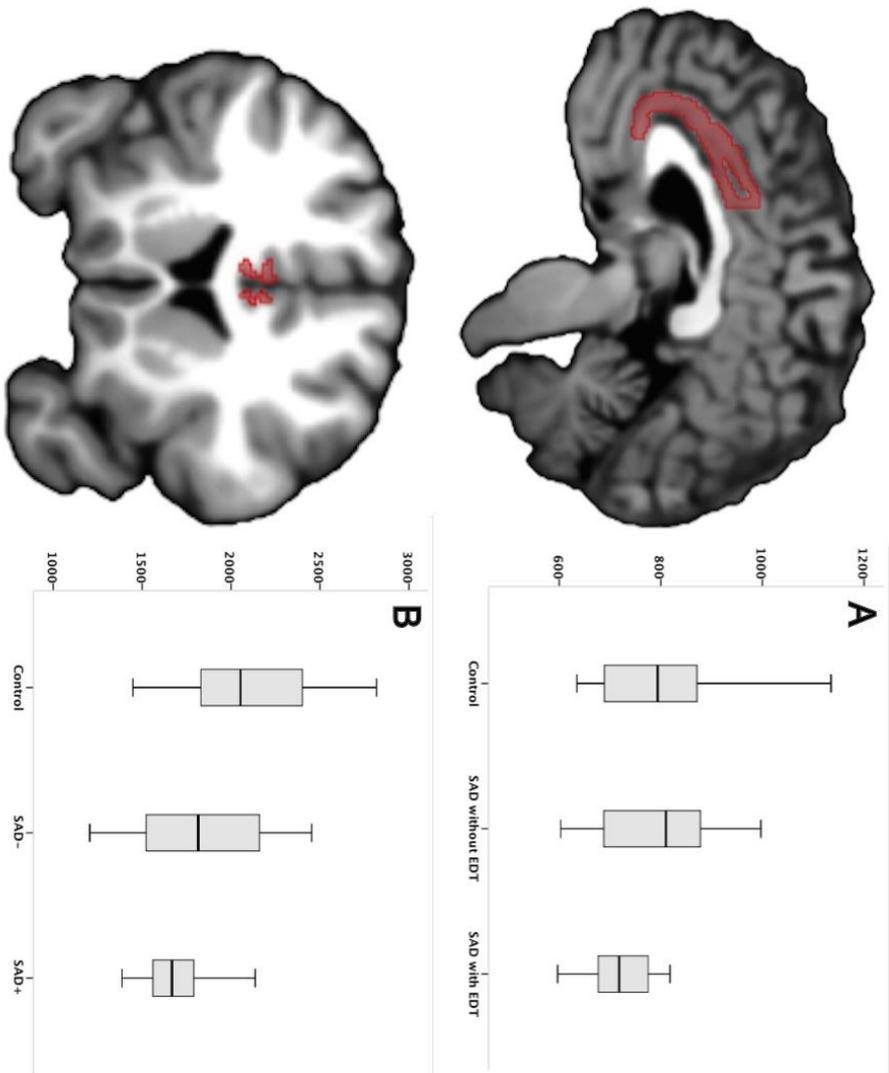
Figure 9: **A.** The left anterior cingulate cortex (ACC) was statistically different between the SAD with EDT group and controls (M =-0.785, 95% HDI [-0.20, -1.33]) and **B.** the right ACC was different between the SAD with EDT group and controls (M = 0.589 95% HDI [0.05, 1.21]).

5.5 *Discussion*

Many studies have focused predominantly on limbic system functioning, and specifically amygdala functioning in SAD. There are few studies, which have explored volumetric abnormalities in SAD (Sareen et al, 2007; Freitas-Ferrari et al, 2010). In addition, there have been no studies to date that have examined differences in grey matter volumes in SAD within the context of EDT compared with individuals with SAD without EDT and controls. This study examined regional grey matter differences in individuals with SAD without EDT, individuals with SAD with EDT and compared findings with healthy controls. We used Freesrufer to examine extracted grey matter volumes in each of the three groups and then to compare differences across the groups using Bayesian statistical inference. The results include a number of noteworthy findings in grey matter volumes in the SAD with EDT group. These included smaller grey matter volumes in the left and right ACC, left thalamus compared with controls and smaller left caudate nucleus volume compared with the SAD without EDT group.

The smaller ACC volume in the SAD with EDT group compared with controls, supports amygdala-frontal network pathology found in other anxiety disorders (Charney & Drevets, 2002). The ACC is an important component of the fear and anxiety circuitry and has a number of connections with other brain regions such as the orbitofrontal cortex, prefrontal cortex and subcortical limbic structures (e.g. the nucleus accumbens and, importantly, the amygdala) (Ressler & Mayberg, 2007; Aupperle & Paulus, 2010). Various subcomponent structures of the ACC also interconnect with areas such as the ventral striatum and insula, both being important components in reward behaviors and interoceptive regulation (Aupperle & Paulus, 2010). Fick et al., (2013), in comparing a matched SAD group (n = 14) with healthy controls (n = 12), observed a negative association between social anxiety symptom severity and the thickness of the right rostral ACC (Frick, 2013). Pannekoek et al (2013) found increased bilateral ACC connectivity in individuals with SAD. ACC connectivity is an important neural correlate in the salience networks in SAD. Network connectivity with the amygdala is important for self-awareness and social fear processing (Pannekoek, et al., 2013). In a study of cortical thickness alterations in SAD compared with healthy controls, Frick et al. (2013) suggest that that decreased ACC volume in SAD may explain the alterations in brain structure in SAD, and

previous findings of dysfunctional regulation and processing of emotion. In a recent meta-anlaytic review of neuroanatomical models of SAD, the ACC emerged as a highly significant structure in SAD pathophysiology and was highlighted as a target structure for future research and clinical investigation (Brühl et al 2014).

The caudate nucleus is a principal component of the basal ganglia and cortico-striatal connections implicated in emotional regulatory processes in many of the anxiety disorders. The caudate nucleus is also the main input region of the basal ganglia. The caudate plays a significant role in voluntary movement and memory formation (Gazzaniga, 2013). Differences in caudate nucleus volume between the SAD with EDT and SAD without EDT groups suggests differences in the pathophysiological mechanisms in SAD. Differences in caudate nucleus functioning has been observed in SAD patients and controls (Åhs et al., 2009). In an fMRI study by Sareen et al (2007), of 10 SAD participants matched with 10 healthy controls, using an implicit sequence learning task, found reduced neural activation of the cortico-striatal circuitry in SAD compared to controls, specifically in the left caudate nucleus (Sareen et al, 2007). The authors concluded that dysfunctional processing in cortico-striatal circuitry was present in SAD. They further speculated that left caudate nucleus dysfunction might be one of the mechanisms to explain the information biases in SAD, specifically that caudate dysfunction might impair action–reward sequencing of social behaviors and the recollection of successful social outcomes in fearful situations in SAD. However, the contribution of EDT was not examined in the aforementioned study. Choi et al (2012) demonstrated in an fMRI experiment involving a variable-length shock anticipation task in 47 individuals, were a shock was delivered with variable length with a corresponding delay during which they would not be shocked. The delay period was cued by a geometric-shaped stimulus indicating whether the particpant was safe (no shock) or in threat (potential shock). They showed involvement of the caudate nucleus in fear and anxiety processing, specifically with regards to threat monitoring and threat cognition (Choi et al., 2012). Meng et al. (2011), in an fMRI task of emotional activation in SAD (n = 16) versus healthy controls (n = 18), found increased activation of the left caudate nucleus, highlighting its involvement in emotional processing in SAD. Another study demonstrated that within the striatum there were dissociable roles of the putamen and caudate nucleus during processing of an instrumental conditioning task, while undergoing fMRI scanning (O'Doherty et al 2004).  O'Doherty et al (2004) found the putamen was involved in prediction of

future reward, whereas the left caudate nucleus was involved in maintaining of action–reward outcome information to enable better responses to be chosen more frequently.

In addition to the observed cognitive processes in the caudate nucleus, increased cerebral blood flow has also been observed in both the caudate nucleus and the ACC in individuals with SAD compared with healthy controls (Åhs et al., 2009; Gaebler et al., 2013). This may be indicative of structural as well as functional changes in the caudate nucleus (Åhs et al., 2009). Psychological stressors including traumatic stressors are known to have a specific effect on the central serotonergic pathways of the brain, which specifically include the caudate nucleus (Waselus et al, 2011). Animal studies have shown that traumatic stressors produce dysregulated serotonergic functioning along multiple neural circuits which include the caudate nucleus, and that prolonged stress may even produce long term architectural changes of the caudate nucleus and related basal ganglia structures (Waselus et al, 2011). This may account, in part, for the smaller caudate volume in the group with SAD and EDT.

Syal et al. (2012) examined cortical and subcortical grey matter differences in 13 SAD participants and 13 age, gender and education matched controls and found no significant grey matter differences between the groups; there were specifically no volumetric differences in corrected amygdala, hippocampal or other cortical grey matter limbic system volumes in their SAD sample. However they did find significantly lower uncorrected cortical grey matter volumes in participants with SAD (Syal et al., 2012). A limitation of their study may have been the small sample size.

5.6 *Conclusion*

Our findings indicate significant brain volumetric abnormalities in SAD with EDT compared with controls.  These volumetric brain abnormalities include smaller left caudate nucleus compared with SAD without EDT and smaller ACC's and left thalamus compared with controls. We believe that the study has sufficient power given the sample size and robust Bayesian statistical analysis in the generation of posteriors for future research. Further, this study marks a novel investigation of the contribution of EDT in the limbic system morphology in SAD. It must be noted that few differences were found between the SAD groups, so it would be difficult to assert

the specificity of thalamic and ACC differences are due specifically to EDT in the pathophysiology of SAD.

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152

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# 6. SINGLE VOXEL PROTON MAGNETIC RESONANCE SPECTROSCOPY ($^1$H-MRS) OF THE AMYGDALA IN SOCIAL ANXIETY DISORDER IN THE CONTEXT OF EARLY DEVELOPMENTAL TRAUMA

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6.1 *Abstract*

Early developmental trauma (EDT) has been hypothesized to play a significant role in the pathophysiology of social anxiety disorder (SAD), specifically through dysregulation of the amygdala. Dysregulated neurometabolism of the amygdala has been found in both SAD and EDT, however there have been no published neurometabolite studies in SAD in the context of EDT. We used single voxel proton magnetic resonance imaging to elucidate the neurometabolite profiles of the left amygdala in 26 individuals with SAD with EDT compared with 20 individuals with SAD without EDT and 22 healthy controls. Bayesian statistical testing was performed to compute between-group differences in probabilities of selected neurometabolites: N-acetyl-aspartate (NAA), myo-inositol or inositol (Ins), glutamate (Glu), and its precursor glutamine (Gln). Differences were found in Ins, NAA and Gln in the left amygdala in the SAD with EDT group compared with the control group. Differences were also found in NAA, Glx in the SAD without EDT compared to the control group. No differences were found between the SAD with EDT and SAD without EDT groups. Distinct neurometabolites were dysregulated in SAD with EDT compared with controls, suggesting specificity in the pathophysiology of SAD with the occurrence of EDT.

Key Words: spectroscopy; ^{1}H-MRS; social anxiety; early life trauma; pathophysiology; neurometabolites

6.2 *Introduction*

Social anxiety disorder (SAD) is classified as an anxiety disorder in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (American Psychiatric Association, 2013). The DSM-5 characterizes SAD as a "marked and persistent fear of social or performance situations in which embarrassment may occur. Exposure to social or performance situations almost invariably provokes an immediate anxiety response" (American Psychiatric Association, 2013). Dysregulated brain energy metabolism and alterations in neuronal plasticity are among the leading hypotheses in the pathogenesis of anxiety disorders, including SAD (Martin et al, 2009; Howells et al, 2015). The lifetime prevalence of SAD is much more common in developed (6.1%) than in developing (2.1%) countries (Stein et al, 2010). In the South African Stress and Health Study (SASH) (Herman et al, 2009) the most prevalent class of lifetime disorders was the anxiety disorders (15.8%) with SAD at 2.8%.

SAD usually begins in early childhood and adolescence, follows a chronic course and can be highly debilitating in terms of academic, work, and social functioning (Schneier et al, 1994). The age of onset for SAD is usually between 12 and 13 years, however evidence suggests that it may be earlier than this (Bandelow & Stein, 2004). SAD also has strong childhood developmental origins (Ollendick, Hirshfeld-Becker, 2002), including exposure to early developmental trauma (EDT) (Kuo et al, 2012). EDT may be loosely defined as any traumatic experience that occurs before 18 years of age (Bernstein, 1997). It has been estimated that, on average, one in ten children annually in the United Sates, has been neglected or psychologically abused and that approximately between 4 -16% have been physically abused (Gilbert et al, 2008). These early developmental traumas constitute a significant aetiological risk for SAD during childhood and in later adult life (Kuo et al, 2012; Erwin et al, 2006).

The contribution of EDT to the aetiology of SAD is not well understood. EDT has been hypothesized to underpin the aetiology of SAD, or at least to play a significant role in the development of SAD in many individuals (Bandelow et al. 2004; Kuo et al 2011). EDT is also believed to significantly influence developing emotional systems of the brain (Heim & Nemeroff, 2001; Holmes et al, 2005). The impact may vary from significant changes in neurological organization of structural neural networks of emotional processing centers, to changes in neurotransmission and neuro-hormone modulation (Heim & Nemeroff, 2001). For example Holmes, et al. (2005) found that

early maternal separation in rodents significantly affected neurotransmitter systems (corticotropin-releasing factor, serotonin, noradrenaline, and glutamate processing and production) implicated in emotional processing. Various neural subsystems that process specific emotions integrate with the limbic system (Zillmer & Spiers, 2001). SAD is thought to be associated with dysregulation of cortico-limbic circuitry, specifically the amygdala (Warwick et al, 2008). The amygdala plays a significant role in fear learning and social fears and is integral to the pathophysiology of SAD (Detweiler, Comer & Albano, 2010). Specifically, individuals with SAD show increased amygdala activity when encountering perceived socially threatening situations (Phan et al, 2006). Furthermore, the amygdala is implicated in fear learning in early traumatization in EDT. However, data on the structural, and neurometabolism of the amygdala in SAD are few (Liao et al, 2011; Talati et al, 2013).

Few studies in SAD have documented alterations in amygdala volume or morphometry (Blackmon et al., 2011; Frick et al., 2013; Talati et al, 2013) and there are few functional magnetic resonance imaging (fMRI) studies (Freitas-Ferrari et al, 2010). Functional activations during social fear tasks have been found to be more consistent in the left compared to the right amygdala (Hardee et al., 2008; Blackmon et al., 2011). Further, in a systematic review and meta-analysis combining results from 54 fMRI and PET studies, Baas et al. (2008) found a common pattern of lateralized amygdala activation; the left amygdala was more often activated than the right amygdala. The amygdala is a highly technically complex target for ^{1}H-MRS due to its small volume, high field inhomogeneity and shared boundary with the hippocampus, with contrasting changes in response to stress (Nacewicz et al, 2012). In addition, few studies have examined neurometabolite dysfunction in SAD (Freitas-Ferrari et al, 2010; Bandelow & Stein, 2004; Howells et al, 2015). Substantial clinical evidence supports the involvement of glutamate (Glu) in the anxiety disorders (Harvey & Shahid, 2012; Cortese & Phan, 2005; Krystal et al, 2010) and there is some evidence for the involvement of associated neurometabolite glutamine (Gln) (Pollack et al, 2008). For example, in a spectroscopy study by Phan et al (2005), patients with SAD demonstrated increased Glu levels in the anterior cingulate cortex (ACC) compared to healthy controls. The authors also found a correlation between the magnitude of the Glu signal and the severity of social anxiety symptoms (Phan et al, 2005). Anxiolytic treatment has been associated with a change in the Glu and Gln

signals over the course of treatment administration, often from a reduction in signal to an increase in signal (Pollack et al., 2008 cited in Harvey & Shahid, 2012). Pollack et al. (2008) observed a higher concentration of Glu and Gln in the whole brain $^{1}$H-MRS analysis of 10 SAD matched participants, and lower concentrations of Glu and Gln in the thalamus after levetiracetam treatment; indicating that treatment may lower Glu and Gln in that brain region (Pollack, 2008). Glu dysregulation in a number of brain regions has also been found in non-human primates exposed to EDT. Some studies have documented an increased Glu signal (Mathew et al, 2003), while other studies have found a decreased Glu signal (Llorente et al, 2012). There is also good evidence that Glu is involved in fear conditioning and in the neurobiological response to aversive stimuli (Harvey and Shahid, 2012). Thus, there appear to be some similarities in the aforementioned neurometabolite profiles in both EDT and SAD and it is plausible that pathophysiological processes underlying amygdala functioning may play a role through EDT as an aetiological precursor of SAD. Other neurometabolites may be involved in amygdala functioning in SAD and EDT.

Myo-Inositol or Ins has been implicated in the pathophysiology of depression and some anxiety disorders (Einat et al, 1999). Ins plays a substantial role in glial metabolism and reductions in Ins are likely to indicate glial loss or altered glial metabolism (Coupland, 2005). In a review of the exogenous administration of Ins, it was reported to be effective in the treatment of a number of psychiatric disorders, such as depression, panic disorder and obsessive compulsive disorder (Levine, 1997; Einat et al, 1999). Reductions in Ins have been found in a number of grey matter structures in psychiatric disorders. For example, lower Ins has been found in the ACC in depressed patients (Coupland et al, 2005), however Ins has not been investigated in the amygdala in individuals with SAD and in individuals with EDT.

NAA, which is almost exclusively found in the nervous system, is a mitochondrial (Baker et al, 2002) and microsomal (Aryannur et al, 2008) amino acid, frequently characterized as a marker of neuronal density and functional integrity (Barker, 2001). Some studies have shown area specific decreases of NAA in the brain in a variety of neuropsychiatric and cognitive disorders (Ferguson et al, 2002; Friedman et al, 1998; Jung et al, 1999; Ariyannur, 2008).

Given the absence of well-established studies detailing neurometabolites within the amygdala in SAD in the context of EDT, we investigated the neurometabolite profile

of the left amygdala in individuals with SAD in the context of EDT. We conducted a cross-sectional proton magnetic resonance imaging ($^1$H-MRS) study of neurometabolites in the left amygdala across three study groups: SAD with EDT, SAD without EDT and a healthy control group. We compared a primary group of participants with DSM 5 generalized SAD with EDT, a group with SAD without EDT and a group of healthy control participants; to identify neurometabolite differences between the groups. It was hypothesized that the SAD with EDT group would have significantly greater neurometabolite differences within the left amygdala than SAD without EDT and controls. We used a Bayesian approach as the principle statistical technique, with a philosophical approach to the data. The popularization of the use of Bayes' theorem in recent years has revolutionized science in fields as diverse cosmology, zoology and cognitive science. Its self-consistent philosophy naturally embodies the scientific method: one has an initial belief, updates that belief by carrying out an objective experiment, gains quantifiable information because of that, and updates one's belief before beginning the next experiment (Kruschke, 2011; Sivia & Skilling, 2006). One does away with the `frequentist' approach in which an ensemble of imaginary experiments is considered and an arbitrary (but conventional) `p-value' is relied upon (Kruschke, 2011).

6.3 *Methods*

6.3.1 *Participants*

The sample (N = 68) comprised individuals with SAD and EDT (n = 26; 38.2%), individuals with SAD without EDT (n = 20; 29.4%) and gender, ethnicity, language, handedness and education matched healthy controls (n = 22; 32.4%).

6.3.2 *Procedures*

Ethical approval for the study was obtained from the Health Research Ethics Committee at the Faculty of the Health Sciences, Stellenbosch University. Written, informed consent was obtained from participants. A clinical psychologist assessed participants for the presence or absence of SAD and EDT, as well for comorbidities. Following a diagnostic assessment, participants attended a follow up visit within two weeks for $^1$H-MRS scanning.

*6.3.3 Demographics and screening*

Participants were screened with the Mini International Neuropsychiatric Interview (MINI) version 6.0 (Sheehan et al., 1998). The primary method of diagnostic assessment was the MINI for diagnosis of SAD. The Liebowitz Social Anxiety Scale (LSAS), which discriminates between generalized SAD and specific SAD, was used with a cut-off score of 60 to assess for inclusion for the SAD groups (Baker et al., 2002; Mennin et al., 2002). Participants were included in the SAD groups if their primary diagnosis was generalized SAD according to criteria on both the MINI and LSAS. The Childhood Trauma Questionnaire Short Form (CTQ-SF) was used to assess for EDT and a cut-off score of 45 was used to establish exposure to EDT. The CTQ-SF (referred in this paper as the CTQ) is a 28 item retrospective measure of childhood trauma, which includes five subcategories of childhood trauma, namely: emotional neglect, emotional abuse, physical neglect, physical abuse and sexual abuse (Bernstein et al, 1994).

Participants with psychotic disorders, bipolar mood disorders, obsessive-compulsive disorder, eating disorders, current alcohol or other drug use disorders, and neurological disorders, were excluded. Participants with past or current major depressive disorder (MDD) were included (See Table 6. for details of percentages of MDD and group differences). Similarly, other disorders as assessed on the MINI were included if they were not primary diagnoses. Participants on psychotropic medications, other than selective serotonin reuptake inhibitors (SSRIs), were also excluded. Participants on SSRIs were requested not to take their medication on the day of the ^{1}H-MRS scan. Exclusionary criteria for the ^{1}H-MRS scans were pregnancy, cardiac pacemaker, metal prostheses or pin(s), clips on blood vessels, inner ear prosthesis, an infusion pump, or a metal intra-uterine contraceptive device.

Table 6

Demographic variables, clinical data and neurometabolite concentrations

| Characteristic | SAD with EDT | SAD without EDT | Control Participants | Group Comparison |
|---|---|---|---|---|
| **Demographic** | | | | |
| Age (y) | 34.2 (SD 9.6) | 33.0 (SD 11.5) | 29.4 (SD 7.3) | |
| Gender (Female/Male) (n) | 16/10 | 11/9 | 13/9 | $\chi^2$ [2] = 0.20 P = 0.91 |
| Years of Education[a] | 14.5 (2.0) | 16.3 (3.2) | 15.5 (2.3) | ANOVA [2, 65] F = 2.81 p = 0.67 |
| Ethnicity (n) | Black 2 Colored 8 White 15 Other 1 | Colored 1 White 18 Other 1 | Black 2 Colored 3 White 17 | $\chi^2$ [6] = 8.93 P = 0.18 |
| **Clinical variables** | | | | |
| LSAS Score | 80 (60-121) | 78 (60-134) | 19 (0-53) | * t[44] = -.24 p = 0.22 |
| Fear Score | 41 (25-60) | 43 (27-71) | 10 (0-26) | * t[44] = .58 p = 0.20 |
| Avoidance Score | 39 (19-64) | 36 (17-63) | 9 (0-27) | * t[44] = -1.00 p = 0.29 |
| CTQ Score | 61 (46-89) | 34 (26-40) | 31 (25-35) | |
| Emotional Neglect | 16 (5-23) | 10 (5-15) | 7 (5-14) | |
| Emotional Abuse | 17 (8-23) | 7 (5-12) | 7 (5-10) | |
| Physical Neglect | 10 (5-21) | 6 (5-13) | 6 (5-10) | |
| Physical Abuse | 10 (5-25) | 6 (5-7) | 6 (5-11) | |
| Sexual Abuse | 9 (5-22) | 5 (5-6) | 6 (5-12) | |
| MDD | | | | |
| Current | 3 (11.5%) | 1 (5.0%) | 0 (0%) | *$\chi^2$ [1] = 0.56 P = 0.46 |
| Past Episode | 13 (50%) | 7 (35.0%) | 1 (4.5%) | *$\chi^2$ [1] = 0.92 P = 0.34 |
| **Neurometabolite concentrations** (mmol/L) | | | | |
| Glutamine | 3.49 (2.36-6.06) | 3.83 (1.15-6.47) | 2.93 (1.52-4.61) | |
| Inositol | 4.94 (3.08-10.10) | 3.55 (1.07-5.34) | 3.06 (1.25-4.21) | |
| N-Acetylaspartate | 6.33 (3.78-7.99) | 6.50 (4.78-8.35) | 7.28 5.59-9.70) | |
| Glx | 10.26(6.01-14.38) | 10.19 (2.18-14.30) | 8.73 (5.88-11.93) | |
| Amygdala Volume | 1605.2 (106-207) | 1626 (1304-1975) | 1692.8 (1351-2277) | |

Note. [a] *Years of education starting from primary school through to tertiary education*. Definitions: MDD = Major Depressive Disorder; Glx = Glutamate and Glutamine. The re were no differences percentage of participants with SAD with EDT and SAD without EDT that were married did not differ by gender, $X^2$(1, $N$ = 46) = 0., $p$ = .35. * These group comparisons were conducted on the SAD groups only; where MDD and LSAS scores were compared.

*6.3.4 Neuroimaging parameters*

Each scanning session lasted approximately one hour. Conventional structural data (for localization purposes) was acquired. A Siemens 3 Tesla Allegra fMRI scanner at the Cape Universities Brain Imaging Centre (CUBIC at Tygerberg Campus) was used to acquire images. A high resolution T1-weighted 3D MPRAGE structural image was first obtained with the following parameters: sagittal orientation, TR =2300ms, TE = 3.93ms, FOV = 220 mm, 160 slices; 256 x 256 matrix; 1 x 1x 1 mm^3 resolution; 9 minute scan time.

In order to acquire the left amygdala volume measurement, we carried out further analysis using Freesurfer, a brain imaging software package designed for the study of cortical and subcortical anatomy (Fischl & Dale, 2000). Freesurfer's processing stream consists of several different stages: volume registration with the Talairach atlas, bias field correction, initial volumetric labeling, non-linear alignment to the Talairach space and a final labeling of the volume (Dale & Fischl, 1999). One of the major benefits of Freesurfer is its ability to carry out all processing steps automatically, however its processing pipeline is computationally intensive; we, therefore, utilized custom batching scripts at the Centre for High Performance Computing (CHPC) in Rosebank, Cape Town (http://www.chpc.ac.za). Intracranial volume was controlled for in analysis of left amygdala volume.

*6.3.5 $^1$H-MRS parameters*

Placement of the amygdala followed left amygdala voxel placement procedures used by Hoerst et al (2010). We firstly performed positioning of the voxel using sagittal MPRAGE slices because of good grey matter/white matter contrast for localizing the amygdala. Secondly, the position was adjusted on T2 sagittal images, avoiding partial volumes of blood vessels or ventricles in the voxel and careful placement along the alveus of the left hippocampus to ensure separation from the hippocampus. Finally the voxel position was verified in the reconstructed transverse and coronal planes (Hoerst et al, 2010) (see Figure 10 *and see* **Appendix C** *for location and placement of the left amygdala voxel*).
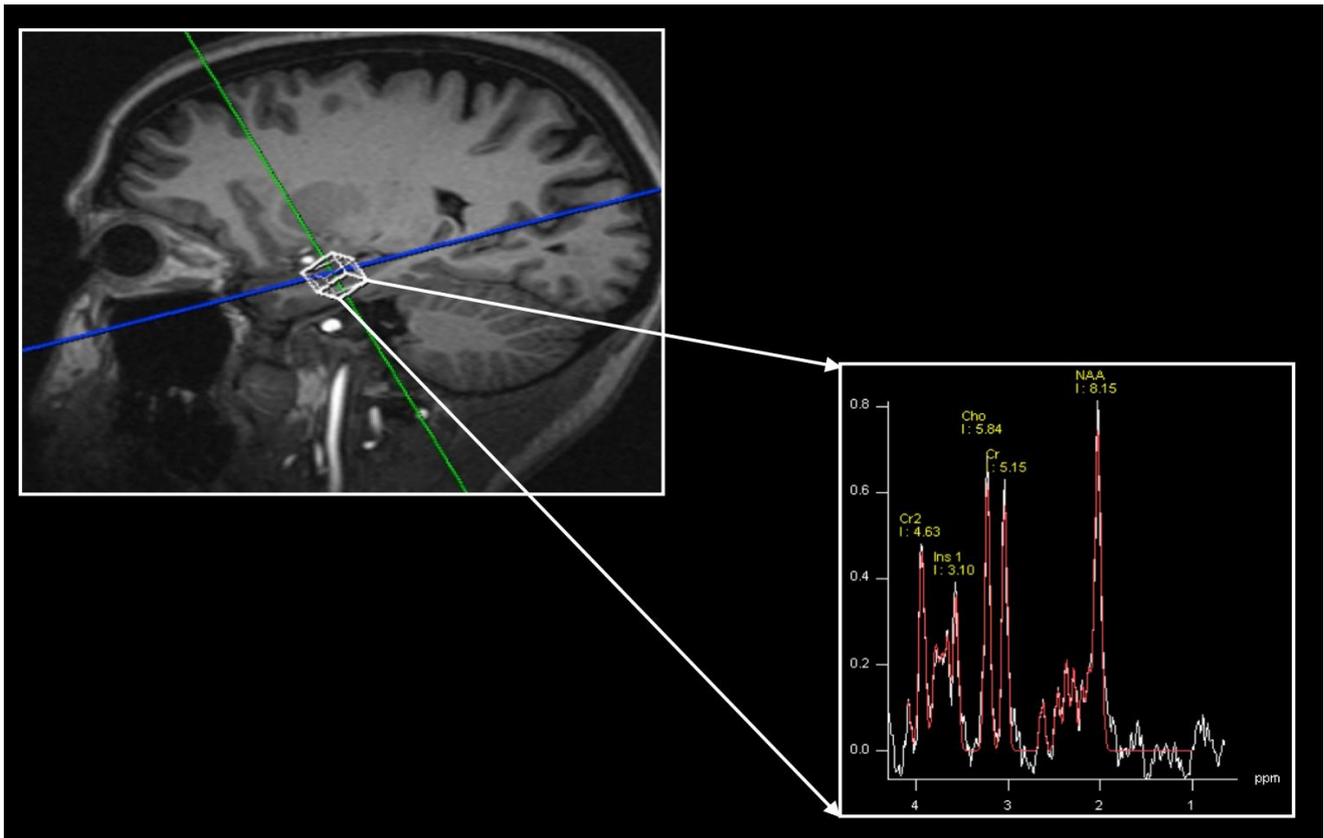
Figure 10. Location of the voxel: A 10x12x12 mm Voxel was placed within
the left amygdala. Representative spectra is shown for the specific voxel.
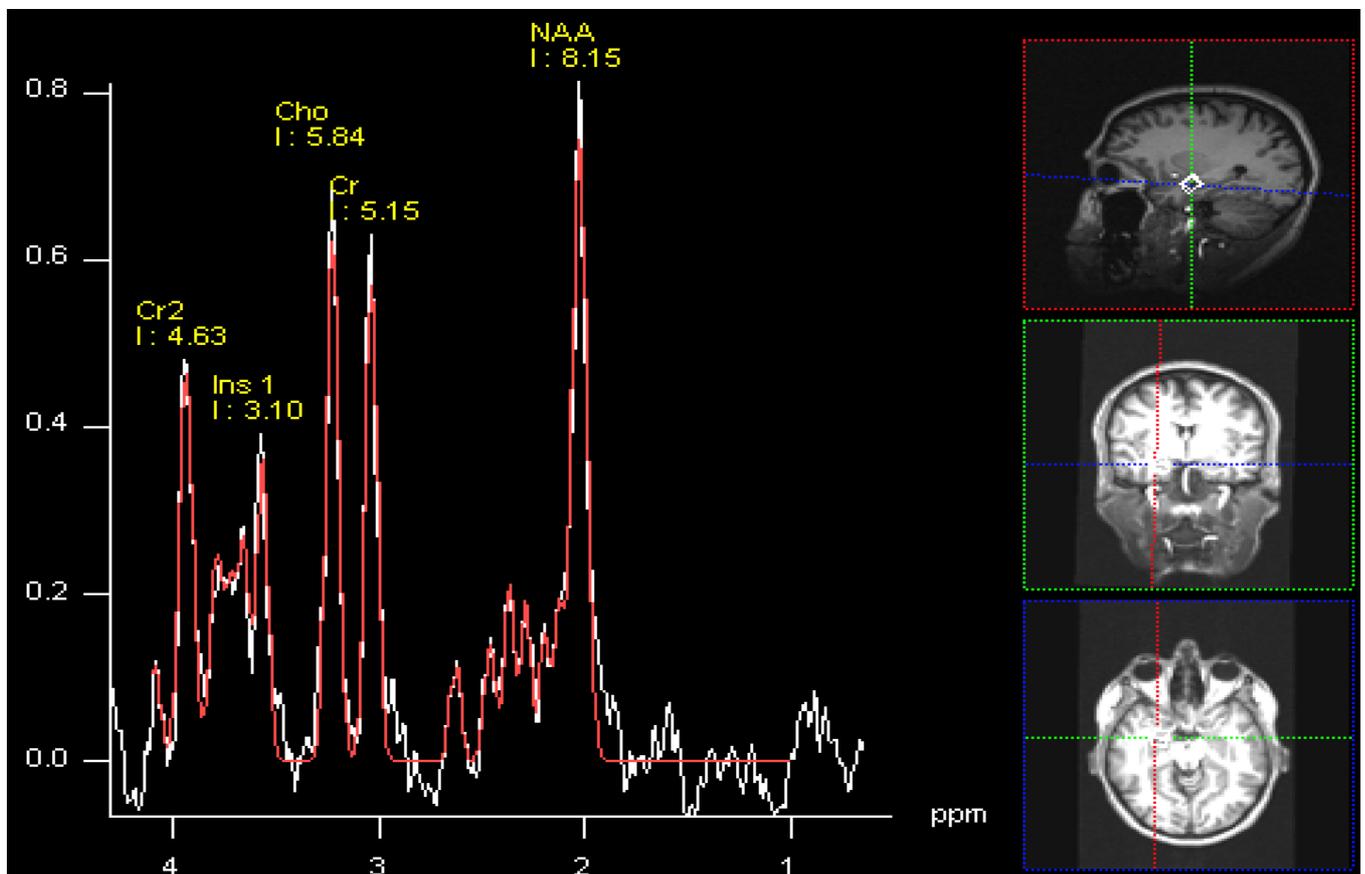


Figure 11. ^{1}H-MRS Spectra: Spectral peaks identified show NAA, Cr, Ins, Cho and NAA. The
other neurometabolite concentrations were extracted from the ^{1}H-MRS spectra using
LCModel post processing software and then further statistical power analysis was performed.

167

The following scan parameters were used: ^{1}H-MRS data for a 10 x 12 x12 mm voxel placed in the left amygdala using an EPI volumetric navigated point-resolved spectroscopy (vNav PRESS) sequence (Hess et al., 2011a) with real-time shim and motion correction (TR = 2000ms, TE = 30ms, 544 averages, vector size 1024, spectral bandwidth 1000kHz). The water unsuppressed  H-MRS measurements were acquired for TE = 30ms (Hess et al, 2011a). Full water saturation was adjusted following automatic shimming and 5 to 10 minutes of manual shimming. ^{1}H-MRS absolute metabolite data were reported in relation to water ($H_2O$). The total ^{1}H-MRS scan time for each participant was approximately 21 minutes. Pre-processing of the water-suppressed data included cross-correlation with a simulated spectrum and singular value decomposition to perform a weighted average of individual measurements in a frequency and phase coherent manner (Hess et al., 2011b; Hess et al 2014). Post processing analysis was performed on the output ^{1}H-MRS Spectra (see Figure 10, Figure 11 and Figure 12 for information on spectra) using LCModel. The reported Cramér-Rao  minimum variance bounds (% Standard deviation or %SD) was used as an exclusion criteria. Neurometabolites were excluded if their %SD fell below 20%. Significance was set at 20% per metabolite. Qualitative analysis entailed the exclusion of participants with poor line-width and signal to noise ratio (SNR); these participants' ^{1}H-MRS scans were not interpretable. Glu and GPC were excluded as they were above the 20 %SD threshold (See Table 7 for more information on LCModel analysis).
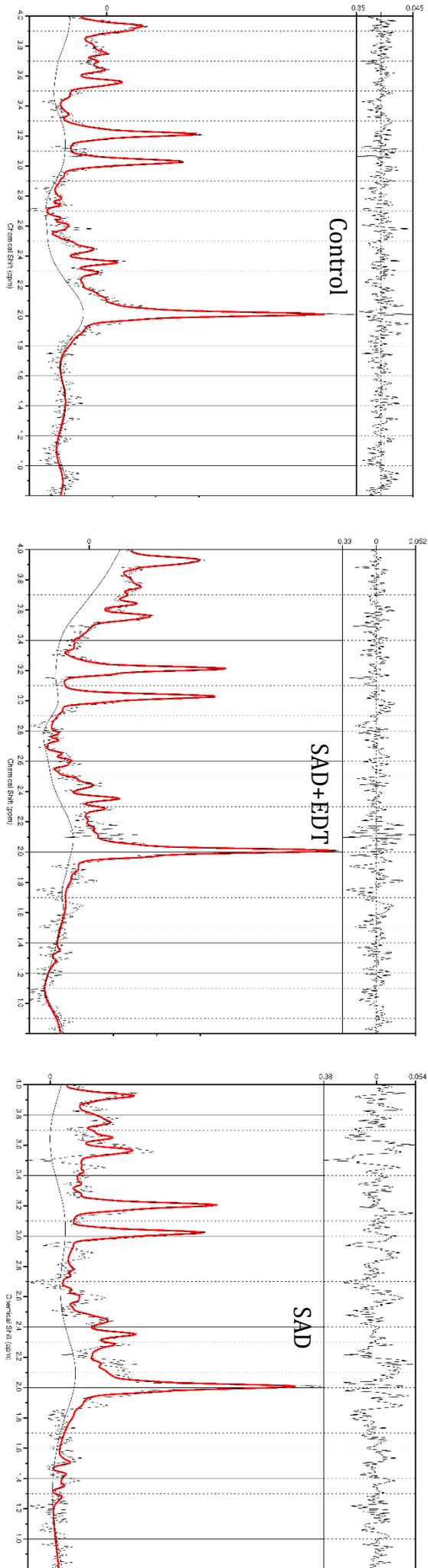
Figure 12. Spectra from LCModel, from left to right of the control group, SAD with EDT group and SAD without EDT group respectively. The spectra were normalized to water unsuppressed, but not for tissue composition.

Table 7

LCModel analysis results demonstrating line-width, %SD and SNR

| Neurometabolite | SAD with EDT | | SAD without EDT | | Controls | | Group Comparisons |
|---|---|---|---|---|---|---|---|
| | %SD Mean | SD | %SD Mean | SD | %SD Mean | SD | |
| Glu | 0.37§ | 0.12 | 0.38§ | 0.21 | 0.46§ | 0.19 | ANOVA [2, 56] F = 0.970 p < 0.39 |
| Gln | 0.17 | 0.03 | 0.24§ | 0.28 | 0.20 | 0.07 | ANOVA [2, 56] F = 1.301 p = 0.28 |
| Ins | 0.13 | 0.04 | 0.19 | 0.12 | 0.19 | 0.08 | ANOVA [2, 56] F = 2.682 p = 0.08 |
| NAA | 0.06 | 0.05 | 0.06 | 0.01 | 0.05 | 0.02 | ANOVA [2, 56] F = 0.671 p = 0.51 |
| GPC | 2.57 | 4.01 | 3.96 | 0.17 | 2.01 | 3.40 | ANOVA [2, 65] F = 0.126 p = 0.88 |
| Glx | 0.16 | 0.04 | 0.19 | 0.19 | 0.18 | 0.04 | ANOVA [2, 56] F = 0.497 p = 0.61 |
| Linewidth | 0.06 | 0.05 | 0.06 | 0.05 | 0.04 | 0.03 | ANOVA [2, 69] F = 0.726 p = 0.49 |
| SNR | 7.42 | 2.75 | 7.32 | 3.02 | 7.91 | 3.44 | ANOVA [2, 69] F = 0.252 p = 0.78 |

Note. * statistical significant difference (p < 0.05). Gln statistically significant difference between groups with regard to %SD. § is above set Cramér-Rao threshold for %SD in LCModel.

Table 8

BEST for neurometabolite differences between the SAD with EDT, SAD without EDT and control groups

| Neurometabolite | SAD without EDT and Controls | | | SAD with EDT and Controls | | | SAD with EDT and SAD without EDT | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | BEST Effect Size | Lower Bound | Upper Bound | BEST Effect Size | Lower Bound | Upper Bound | BEST Effect Size | Lower Bound | Upper Bound |
| Gln | 0.77* | 0.11 | 1.52 | 0.58 | -0.13 | 1.22 | 0.36 | -1.05 | 0.34 |
| Glx | 0.73** | 0.026 | 1.53 | 0.77** | 0.086 | 1.48 | 0.076 | -0.77 | 0.62 |
| Ins | 0.52 | -0.20 | 1.16 | 1.21** | 0.496 | 1.96 | 0.91* | 0.15 | 1.54 |
| NAA | -0.69** | -1.4 | -0.04 | -0.81* | -1.52 | -0.17 | -0.17 | -0.79 | 0.52 |
| Amygdala | 0.22 | -0.42 | 0.87 | 0.38 | -0.25 | 1.01 | 0.37 | -0.25 | 1.01 |

Note. * Statistical difference according to BEST with ROPE > 0.1. ** Statistical difference, yet ROPE < 0.1. Definitions: Gln = Glutamine; Glx Combined Glutamate/Glutamine signal; Ins = Inositol; NAA = N-Acetylaspartate. Other neurometabolites were excluded via LCModel post-processing. Only the listed Neurometabolites were included in the BEST analysis.

171

*6.4 Data analysis*

For the reporting of demographic variables and analysis of LCModel results;
ANOVAs and t-tests were used.  Bayesian statistical data analyses were computed
following LCModel analysis to determine group differences for significant
neurometabolites. Analysis LCModel output indicated that there were no significant
differences between neurometabolites between groups (see Table 7). Glu, GPC,
NAAG, and PCh were excluded from the Bayesian statistical analysis as the mean
%SD was above 20%. Bayesian statistical analysis was chosen due to the potential
problem of null hypothesis significance testing (NHST) in the assumption that the
mean neurometabolite concentration is the same (H0) for the control and SAD (or
SAD with EDT) groups, or that the latter has a greater mean than the control group
(H0). NHST differences were computed for clinical variables and neurometabolites.
We evaluated this numerically with the *Bayesian estimation supersedes the t test*
(BEST) using the Markov Chain Monte Carlo (MCMC) algorithm (Kruscke, 2013,
2013), effectively simulating three datasets for each hypothesis and comparing their
relative probabilities. An equiprobable prior was chosen for the model assuming both
H1 and H0 being true. The MCMC method used was an adaptive Metropolis-within-
Gibbs sampler (Roberts & Rosenthal, 2009; Kruscke, 2013). A mean credible value
was taken as an estimate of the actual difference and a 95% *Highest Density Interval*
(HDI) was taken as the range where actual differences had 95% credibility. Every
sample consisted of a burn-in period of 10 000 iterations or simulated experiments,
based on the standardized model parameters suggested by BEST (Kruschke, 2013)
producing a total of 100 000 samples.

The model was checked for convergence by considering the relevant kernel density
plots and was also checked diagnostically through autocorrelation plots within the
python BEST program; which showed throughout the iterative process that the
autocorrelation was satisfactorily reduced to a nominal amount. The 95% highest
density intervals (HDI) of the posterior distributions were used to describe the
credible interval for each of the parameter estimates. For more details on the BEST
algorithm we refer the reader to www.indiana.edu/~kruschke/BEST. We used the
python version pyBEST available from https://github.com/strawlab/best. We further
defined a region of practical significance (ROPE) (Kruschke, 2012) in order to further
estimate whether the probability of the difference was too small to matter. We used a

difference of ± 0.1 (Krischke, 2012) on the MCMC - HDI output in order to estimate whether the probability that the true values lie within 0; which would indicate a difference between the groups that would be too marginal to matter. The reason for selecting the current ROPE value was based on selecting a conservative estimate for likelihood of estimating probabilities (Kruschke, 2013), given that we know very little about the neurometabolite differences in the amygdala in the aforementioned groups.

*6.4.1 Descriptive statistics computations*

SPSS 19.0 was used to obtain descriptive statistical data and to test for kurtosis and homogeneity of variance. Post-hoc NHST pairwise comparisons were computed for clinical variables, to examine %SD significance in LCModel within groups and descriptive statistics for neurometabolites.

6.5 *Results*

*6.5.1 Descriptive data*

See Table 6 for a description of the demographic, clinical and neurometabolite data for each of the three groups. In terms of comorbidity, 3 participants (11.5%) with SAD with EDT, one individual (5.0%) with SAD without EDT had major depression (MDD). Approximately 13 (50.0%) individuals with SAD with EDT and 7 with SAD without EDT (35.0%) and one control participant (4.5%) reported a past history of at least one episode of MDD, occurring more than 2 weeks prior to their initial assessment. There were no differences in the rate of MDD (MDD past episode p = 0.78 and MDD current p = 0.14) or mean LSAS scores (p = 0.22) between the SAD with EDT and SAD without EDT groups (See Table 6 for details).

*6.5.2 Inferential Data*

BEST results were computed and presented separately for each neurometabolite that could be analysed post LCModel analysis. Table 8 presents the BEST results for analysable neurometabolites extracted from the LCModel post-processing step (*see also* **Appendix D** *for detail on each of the BEST output graphs*). We now discuss neurometabolite differences across the three groups.

*6.5.2.1 Glutamate and glutamine*

There was credible difference in Gln in the SAD without EDT group compared with controls (BEST Effect Size 0.579, 95% HDI [0.112, 1.52]). The BEST differences in

173

Glx in the SAD without EDT group (BEST Effect Size 0.768, 95% HDI [1.48, 0.0861]) and the BEST differences in Glx in the SAD with EDT group (BEST Effect Size 0.725, 95% HDI [0.0256, 1.53]) compared to controls.

### 6.5.2.2 N-Acetylaspartate

There was a statistically significant differences in the SAD with EDT (BEST effect size -0.0422, 95% HDI [-1.52, -0.165]) and SAD without EDT (BEST effect size -0.0422, 95% HDI [-1.52, -0.165]) groups in NAA compared with the control group (BEST effect size -0.0422, 95% HDI [-1.52, -0.165]).

### 6.5.2.3 Inositol

Ins was higher in the SAD with EDT group compared with controls (BEST effect size 1.21 95% HDI [0.496, 1.96]).

### 6.5.2.4 Amygdala volume

There were no significant group differences according to BEST in left amygdala volume across the groups (See Table 8 and **Appendix D** for details).

### 6.6 Discussion

No published studies have examined neurometabolite profiles in SAD in the amygdala. This study compared differences in neurometabolites in the left amygdala of individuals with SAD with EDT, individuals with SAD without EDT and healthy controls, using $^1$H-MRS. A combination of NHST and Bayesian statistical analysis was undertaken to address group differences. A number of noteworthy differences in the left amygdala were found across the groups. Firstly, there was lower concentrations of NAA in both the SAD with EDT and in SAD without EDT groups compared to controls. There was also elevated Ins in the SAD with EDT group compared with controls. There was elevated Gln in the SAD without EDT group and elevated Glx in both the SAD with EDT and SAD without EDT groups.

Of the various neurometabolites subjected to in vivo investigation, it appears that brain Glu has been of specific interest and the most extensively studied in SAD (Phan et al, 2005; Pollack et al, 2008). Phan et al (2005) reported higher Glu ratios in individuals with SAD, in the ACC, compared with matched controls. Phan et al

174

(2005) also found that the Glu ratio was positively correlated with the intensity of SAD symptoms. The ACC contains a vast array of limbic circuitry connections which include the amygdala. There is also high functional connectivity between the ACC and amygdala (Weber et al, 2013). Glu and Gln are co-metabolites. Gln is a precursor of Glu that is mostly found in astrocytes, and is a key component in the neurometabolic cycle of Glu. Our findings of elevated levels of Gln in the amygdala of individuals with SAD without EDT supports dysregulation of emotional processing systems in SAD. There were no Glx differences between the two SAD groups.  It can, therefore, be assumed that the presence of EDT does not account for the mechanisms associated with Gln and Glx neurometabolism. There is currently little evidence for dysregulated Glx or Gln neurometabolism in the amygdala due to early developmental trauma. For example, one of the only studies to investigate this, a non-human study of early developmental trauma and stress, found that prolonged stress in young rats produced a decreased glutamate and glutamine cortical signal with no statistically significant raised levels in subcortical regions, such as the amygdala (Knox et al, 2010). Our current findings, therefore, support the aforementioned finding as well as support studies demonstrating dysregulation or raised concentrations of Glx in various subcortical structures in anxiety disorders and SAD (Phan et al, 2005; Simon & Gorman, 2006; Karl & Werner, 2010).

Elevated levels of Ins were found in the SAD with EDT group, suggesting dysregulated glial metabolism and/or glial loss (Coupland et al, 2005). EDT may have a mediating role, as there were no differences found in the SAD without EDT group compared to controls. There were also no differences in Ins between the two SAD groups, which makes it harder to establish the specific role of EDT in Ins dysregulation in SAD within the context of EDT.  According to Coupland et al. (2005) Ins does not seem to be consistently dysregulated across the brain and only some cortical regions have shown reductions in major depressive disorder and bipolar mood disorder (Coupland et al, 2005). Some in vivo studies have shown corresponding lower NAA and higher Ins levels in disorders with pathology involving neuronal loss and/or gliosis (Kantarci et al, 2004; Coupland et al, 2005). Dysregulated levels of Ins have been associated with astroglial deficits due to early life stress, as demonstrated by Zhang et al (2013), who found dysregulated Ins in traumatic stress induction of rat pups. Ins and NAA are both neurometabolites involved in cellular

175

integrity. Zhang et al (2013) also found dysregulated NAA in a number of brain circuits due to EDT.

We found lower NAA in both the SAD with EDT and the SAD without EDT groups compared to the control group. However no differences appeared between the two SAD groups. Regional reductions in NAA have been argued to represent an in vivo signal of compromised neuronal integrity or fitness (Barker, 2001; Bertolino et al, 1997, 2002). The decreased NAA found in the left amygdala in the SAD with EDT group correlates to what has been found in other limbic system circuitry in animal studies.  Those studies reflect a reduction in NAA and related neuronal density and functional integrity due to early life stress (Coplan et al, 2010; Mathew et al, 2003). This is consistent with findings in other fear and anxiety induction studies (Zhou et al, 2012). For example, Mathew et al (2003) in a study of bonnet macaques, found a decrease in the NAA in comparison to Cr in the ACC in individuals with significant early stress. In a posttraumatic stress disorder (PTSD) study, participants exhibited approximately 30% less hippocampal NAA than healthy controls, despite there being only a 5% hippocampal volume difference between the groups (Schuff et al, 2001; Freeman et al, 1998). In the present study, the SAD with EDT group displayed a greater mean reduction in NAA than the SAD without EDT group, although this was not statistically different between the SAD groups.  Compromised NAA may represent a more generic neuronal marker of anxiety and traumatic stress, without specificity for SAD (or SAD with EDT).

There are very few multimodal neuroimaging studies that have investigated volume differences as well as neurometabolites in SAD or in EDT (Freitas-Ferrari et al, 2010). We examined amygdala volume in order to compare differences of left amygdala proportion together with neurometabolite profiles. However we found no probable difference or significant difference in amygdala volume in the left hemisphere between the three groups. With regard to the SAD with EDT group specifically, these findings do not approximate other findings of reduced amygdala volume in the left hemisphere in individuals with experiences of traumatic stress (Weber et al, 2013). Reduced hippocampal volume and reduced volumes in related limbic system structures have been found in individuals who have experienced traumatic stress (Shu, et al, 2013; Liu et al, 2011). In a study of the relationship between amygdala volume and PTSD symptoms, reduced bilateral amygdala volume

176

was associated specifically with the development of PTSD (Rogers et al, 2009). Volumetric changes in the amygdala have not been consistently observed in studies of SAD, and one of the only studies to date to explore volumetric shifts in the amygdala, by Machado-de-Sousa et al. (2010), indicated an increase in bilateral amygdala volume compared with controls, but the authors found no amygdala changes in a sub-threshold  SAD group. A reduction in amygdala size may, therefore, relate more specifically to the pathophysiological mechanisms associated with PTSD rather than SAD, even in the context of early traumatic events. Shucard et al (2012) elaborated on this by indicating that amygdala and other limbic structure volumes, including the ACC, were negatively correlated with the severity of current traumatic symptom severity, with greater trauma symptoms corresponding to a reduction in amygdala volume. Changes in grey matter density have only been observed in the right amygdala in individuals with SAD, and no left amygdala shifts or changes in volume have been observed (Meng et al, 2013). Amygdala volume correlated weakly and negatively with Gln and weakly and positively with NAA. These correlations have been similarly observed in a study of PTSD patients who exhibited approximately 30% less hippocampal NAA and a 5% reduction in hippocampal volume difference compared with healthy controls (Schuff et al, 2001; Freeman et al, 1998).

The amygdala is a particularly difficult area of the brain to scan using ^{1}H-MRS (Nacewicz et al, 2012); making the average scan time very long and also significantly affecting the overall success rate of scans. There have been few ^{1}H-MRS studies in SAD (Freitas-Ferrari et al, 2010), but of those that have been performed, our findings approximate closely to what has been observed in other subcortical grey matter structures. For example, our findings of higher concentrations of Glx and Ins approximate those of an earlier ^{1}H-MRS study conducted by Tupler et al, (1997); who undertook a treatment study of clonazepam in 19 individuals with SAD matched with 10 controls, and found increased concentrations of NAA, Ins, Glu and Cr in subcortical grey matter in the ACC (Tupler et al, 1997).

### 6.7 *Conclusion*

The neurometabolite profiles of SAD with EDT and SAD without EDT are distinct suggesting that EDT may play a specific role in the pathophysiology of SAD and may modify the neurometabolic profile of the amygdala. Aside from differences in the SAD with EDT and SAD without EDT groups compared with the control group, our

findings also support previous findings for neurometabolite differences in subcortical grey matter structures in SAD, such as raised Glx, Gln, Ins and reduced NAA. There are a number of limitations that warrant mention. Firstly, the interpretation of findings is limited by the relatively small sample size. Further investigation in a larger sample size is needed to confirm these findings. Secondly, we did not use an EDT exposed control group for comparison, which may limit the comparisons made and the extrapolation of findings between the SAD groups. Third, the study included participants currently on SSRI medications, (although use of other medication counted toward being excluded). SSRIs have an effect on neurometabolite ratios (Pollack et al, 2008; Lyoo & Renshaw, 2001) and in this study may have had an effect on neurometabolite ratios of both the SAD with EDT and SAD without EDT groups. However it should be noted that there was no difference between SAD groups in the rate of past and current MDD. Fourthly amygdala placement did not consider the differences of tissue composition and other adjacent structures of the amygdala. Lastly the study could not conclude the specificity of EDT on neurometabilite dysregulation in SAD within the context of EDT, as there were no differences between the SAD groups. This would be worth investigating in furutre research in a follow up study examining further SAD in the context of EDT. An advantage of our study was the combined use of statistical measures such as NHST and Bayesian analysis. A Bayesian approach allowed us to generate a set of posteriors for future research in this area. We recommend that future studies examining SAD and EDT use these posteriors as a means of further examining the evidence obtained in this study.

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187

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# 7. GENETICS, GRAPH ANALYSIS AND CORRELATIONS OF NEUROIMAGING AND NEUROCOGNITIVE DATA

**Previous chapters present findings for the primary study hypothesis, consisting mainly of univariate group differences in neurocognition, grey matter volumes (sMRI), and neurometabolites ($^{1}$H-MRS). This chapter presents a summary of these findings and provides NHST and Bayesian statistical data integrating neurocognitive variables, and sMRI brain volumetric data and $^{1}$H-MRS data for the amygdala. Genotyping analyses are also presented.**

## 7.1 *Introduction*

We have thus far used Bayesian statistics to examine group differences in structural neuroimaging data, neurometabolite profiles and neurocognitive performance. A number of differences in clinical, genetic and neuroimaging data were found between the SAD with EDT group and controls and the SAD without EDT group and controls, while relatively few differences between the SAD with EDT and SAD without EDT groups. (*See* **Appendix E**, *for further details on the comparisons between the SAD group findings from pairwise statistical analysis).*

## 7.2 *Summary of probable group differences from Bayesian comparison statistics*

The following probable statistical group differences in neurocognition were observed in Bayesian BEST analyses: firstly, the SAD without EDT group performed more poorly on the WMS logical memory immediate recall, HVLT immediate recall and HVLT delayed recall compared with controls (pairwise differences in Baysian analysis); secondly, the SAD with EDT group performed more poorly on the WMS logical memory immediate recall, WMS logical memory delayed recall, HVLT immediate recall, HVLT delayed recall, Grooved Pegboard (GPB) dominant hand, GPB  non-dominant hand, Stroop Color, Stroop Word/Color, WCST non-perseverative errors and WCST conceptual level responses compared with controls (pairwise differences); and thirdly, there were no differences on any of the neurocognitive variables between the SAD with EDT and SAD without EDT groups. See chapter 4 for more information on neurocognitive differences.

There were also statistical differences in neurometabolite concentrations (^{1}H-MRS), with increased Inositol (Ins) and Glutamine/Glutamate (Glx), and decreased N-Acetylaspartate (NAA) and Phosphocreatine, (PCr) in the SAD with EDT group compared with controls. Increased concentrations of Glutamine (Glu) and Glutamine/Glutamate (combined signal - Glx) was observed in the SAD without EDT group compared with controls. In addition increased Ins was observed in the SAD with EDT group compared with the SAD without EDT groups. See chapter 6 for more details.

There were no sMRI grey matter differences observed between the SAD without EDT group and controls. Between-group differences were observed with lower grey matter volumes in the left and right ACC and the left thalamus in the SAD with EDT group compared with controls. Lower grey matter volumetric difference was observed in the left caudate in the SAD with EDT group compared with the SAD without EDT group. See chapter 5 for more information.

### 7.3 Descriptive statistics

Participant characteristics are presented in Table 9; much of this data was presented in chapter 5. However clinical variables are included here to account for the possible contributory effects of comorbidity and other demographic variables to group differences. There were no statistically significant group differences in comorbidity (current episode of major depression, past episode of major depression and generalized anxiety disorder), as well as no significant differences on any of the demographic variables (such as age, education, ethnicity and handedness).

Table 9

Total sample characteristics

| Characteristic | SAD with EDT | SAD without EDT | Control Participants |
|---|---|---|---|
| Age (y) | 37(20-62) | 33 (21-59) | 30 (22-47) |
| Gender (Female/Male) (n) | 14/9 | 11/11 | 12/13 |
| Years of Education [a] | 14 (10-19) | 16 (12-23) | 15 (11-19) |
| Ethnicity (n) | Black 1<br>Colored 6<br>White 15<br>Other 1 | Colored 2<br>White 20 | Black 3<br>Colored 3<br>White 19 |
| LSAS Score | 80 (-121) | 78 (60-134) | 20 (0-53) |
| Fear Score | 41 (25-60) | 43 (27-71) | 11 (0-26) |
| Avoidance Score | 39 (19-64) | 36 (17-63) | 10 (0-27) |
| CTQ Score | 63 (44-92) | 35 (26-39) | 31 (25-49) |
| Emotional Neglect | 16 (5-24) | 10 (5-16) | 7 (5-14) |
| Emotional Abuse | 17 (8-24) | 7 (5-13) | 7 (5-10) |
| Physical Neglect | 9 (5-21) | 6 (5-13) | 6 (5-11) |
| Physical Abuse | 11 (5-25) | 7 (5-13) | 6 (5-13) |
| Sexual Abuse | 9 (5-25) | 5 (5-8) | 5 (5-12) |
| Psychiatric Diagnosis | | | |
| Current MDD | 4 | 2 | 0 |
| Past MDD | 11 | 11 | 3 |
| GAD Current | 7 | 6 | 0 |

*Note:* Current MDD = Major depressive disorder, indicating the number of participants in the study with a current diagnosis.  Past MDD = a previous history of major depressive disorder, an episode occurring more than two weeks before diagnosis and likely 6 months prior. GAD Current = a current comorbid diagnosis of generalized anxiety disorder that is not accounted for by SAD presentation. [a] = Years of education starting from primary school through to tertiary education

*7.4 Correlations*

Correlational analysis was undertaken (see Table 10) to further explore the relationship between neurocognitive deficits and volumetric sMRI brain data from pairwise group comparisons. We decided to use NHST for correlations between neurocognitive and sMRI data. NHST Spearman correlations were calculated for statistically significant neurocognitive and sMRI data, which included the left thalamus, left and right ACC and the left caudate nucleus in the SAD with EDT group. However, even though the left amygdala volume was not significantly different across the groups, we included it in the analysis, as it was a region of interest in this study. Correlations were run independently for each group: SAD with EDT, SAD without EDT and controls. An *a priori* power analysis was conducted on the model in order to confirm power in this exploratory analysis. Regression analysis could not be performed because of the small sample size and it was estimated that the Bayesian regression models were undermined by the small sample size and the number of covariate and predictor parameters needed for model selection. An post hoc power analysis showed that there was insufficient power (N = 70 [sample from sMRI data and neurocognitive data sets]) to detect a medium-sized effect; actual power of 0.66 (Critical F = 2.35), with ideal power (1 - β) set at .80 and α = .05, two-tailed, and the power analysis set at a small-to- medium effect size of 0.15 for power analysis (*See* **Appendix F** *for graph of power analysis*). Thus, only correlational analysis was undertaken. Each correlational result is reported below under the appropriate neurocognitive domain:

Table 10

Spearman correlations between neurocognitive and sMRI data in SAD with EDT and SAD without EDT groups

| Correlations in SAD without EDT | | | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| **Neurocognitive test** | **Caudate(L)** | **ACC(L)** | **ACC(R)** | **Amygdala(L)** | **Amygdala(R)** | **Thalamus(L)** |
| WMS immediate | -0.206(.384) | .503(.020)* | .235(.292) | -.170(.449) | -.133(.554) | -.007(.977) |
| HVLT immediate | -.137(.565) | .195(.398) | .285(.199) | -.325(.140) | -.305(.167) | -.150(.516) |
| HVLT delayed | -.308(.186) | .418(.059) | .456(.033)* | -.283(.202) | -.397(.068) | -.083(.720) |
| Correlations in SAD with EDT | | | | | | |
| **Neurocognitive test** | **Caudate(L)** | **ACC(L)** | **ACC(R)** | **Amygdala(L)** | **Amygdala(R)** | **Thalamus(L)** |
| WMS immediate | .008(.970) | -.313(.155) | .197(.392) | .155(.481) | -.300(164) | .013(.954) |
| WMS delayed | .018(.934) | -.187(.403) | .352(.118) | .147(.502) | -.330(.124) | .024(.914) |
| HVLT immediate | -.199(.362) | -.155(.610) | -.064(.783) | .021(.922) | -.059(.791) | -.392(.064) |
| HVLT delayed | -.177(.594) | -.215(.337) | .080(.732) | .012(.956) | -.050(.821) | -.237(.277) |
| GPB dominant | -.434(.039)* | .057(.800) | -.206(.370) | -.171(.434) | .258(.235) | -.073(.741) |
| GPB non-dominant | -.210(.336) | .162(.472) | -.324(.152) | -.302(.161) | .168(.444) | -.068(.756) |
| Stroop Colour | .006(.978) | -.016(.943) | -.026(.910) | .163(.456) | -.065(769) | -.366(.086) |
| Stroop Word/Colour | .119(.588) | .087(.700) | -.034(.885) | .251(.249) | .136(.535) | -.037(.865) |
| WCST NPE | .102(.645) | .187(.403) | .064(.783) | .220(.313) | .427(.042)* | .104(.637) |
| WCST CLR | -.301(.163) | -.196(.383) | -.024(.918) | -.216(.321) | -.436(.037)* | .018(.935) |

*Note*. These correlations were only run the findings from the pairwise comparisons that were statistically different between the groups. This was an intentional focus as to further examine the relationship between grey matter differences and neurocognition. Abbreviations: WMS: Wechsler Memory Scales, HVLT = Hopkins Verbal Learning Test; GPB = Grooved Pegboard; WCST = Wisconsin Card Sorting Test; ACC = Anterior Cingulate Cortex All brain volumes (Caudate; ACC; Amygdala; and Thalamus are measured in mm$^3$)
 * = P < 0.05, are all statistically significant.

### 7.4.1 Verbal memory correlations

In the SAD without EDT group there was a moderate positive correlation with the Wechsler memory scales logical memory immediate recall test and the left ACC (r = .503, p = 0.020). We found no correlations in the SAD with EDT group in the WMS logical memory immediate recall test and brain volumes. In the SAD without EDT group there was a moderate positive correlation with the HVLT logical memory delayed recall test and the right ACC (r = .456, p = 0.033). We found no correlations in the SAD with EDT group in the WMS logical memory delayed recall test and brain volumes.

### 7.4.2 Motor function correlations

In the SAD with EDT group there was a moderate negative correlation with the GPB dominant hand test and the left caudate nucleus (r = -.434, p = 0.039). No significant correlations were found in the GPB non-dominant hand and grey matter volumes in the SAD with EDT group.

### 7.4.3 Executive functioning correlations

No significant correlations were found in the Stroop tests (colors and colors/words) and grey matter volumes in the SAD groups. In the SAD with EDT group we found correlations in the WCST non-perseverative errors and the right amygdala (r = -.427, p = 0.042) and the WCST conceptual level responses and the right amygdala (r = .436, p = 0.037).

*7.5 Graph theoretical analysis (GAT) of sMRI brain volume data*

Univariate analysis of group differences found specific grey matter differences in the SAD with EDT group compared to the control and SAD without EDT groups. We conducted a further analysis of grey matter differences, across diffuse neural networks using graph theoretical analysis (GAT) tools (see Figure 13 and Figure 14). This was conducted in order to examine the specificity of differences between the SAD groups. Only partial GAT analysis was conducted, up until just before full network hub analysis, to establish specificity of grey matter differences. The GAT analysis found marginal differences between grey matter structures and a number of network nodes. We used the node network analysis to differentiate grey matter network structure differences between the SAD with EDT and SAD without EDT groups. We also conducted an analysis of regional network measures of between-ess and found differences suggesting smaller ACC, right cingulate cortex, right occipital lobe, right occipito-temporal lateral fusiform thickness and right orbital-H-shaped thickness in the SAD with EDT group compared with the SAD without EDT group. In particular, these findings support the ACC as an important component structure in the pathophysiology of SAD within the context of EDT. There is suggestion of specificity of neural network functioning involving ACC interconnectivity in the SAD with EDT group compared with both the SAD without EDT and control groups.

Using the GAT approach is based on the assumption that positive correlations between morphometric/volumetric parameters of different brain regions are an indication of connectivity (Bernhardt et al., 2008; Hosseini, et al, 2012). Previous studies have revealed that some of the tractography maps obtained from diffusion tensor imaging are strikingly similar to the pattern of correlations in cortical thickness produced by GAT analysis (Hosseini, 2012). The present study investigated between-group differences in regional network measures, specifically nodal between-ness, on networks threshold-ed. It is however important to note that none of these regions survived after correction for multiple comparisons ($P < 0.05$), therefore we caution against the interpretation of these findings, as the analysis was exploratory in its nature.
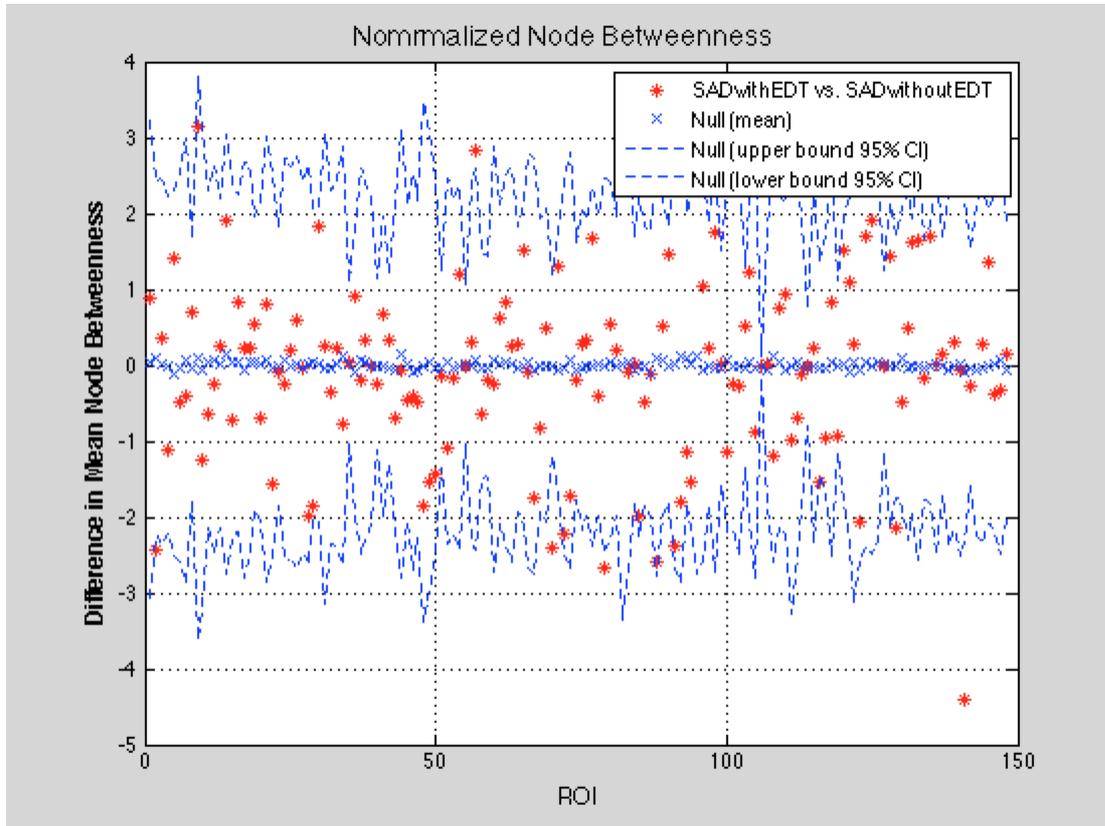
Figure 13 represents Normalised between Node differences between all grey matter volumes from Free Surfer data between the SAD with EDT and SAD without EDT groups. Significant differences set at P < 0.05 for target structures, were found between ACC volumes between the two SAD groups. The ACC was an important area of focus in statistcial analysis and Node analyses was used to further examine these differences between the SAD groups.
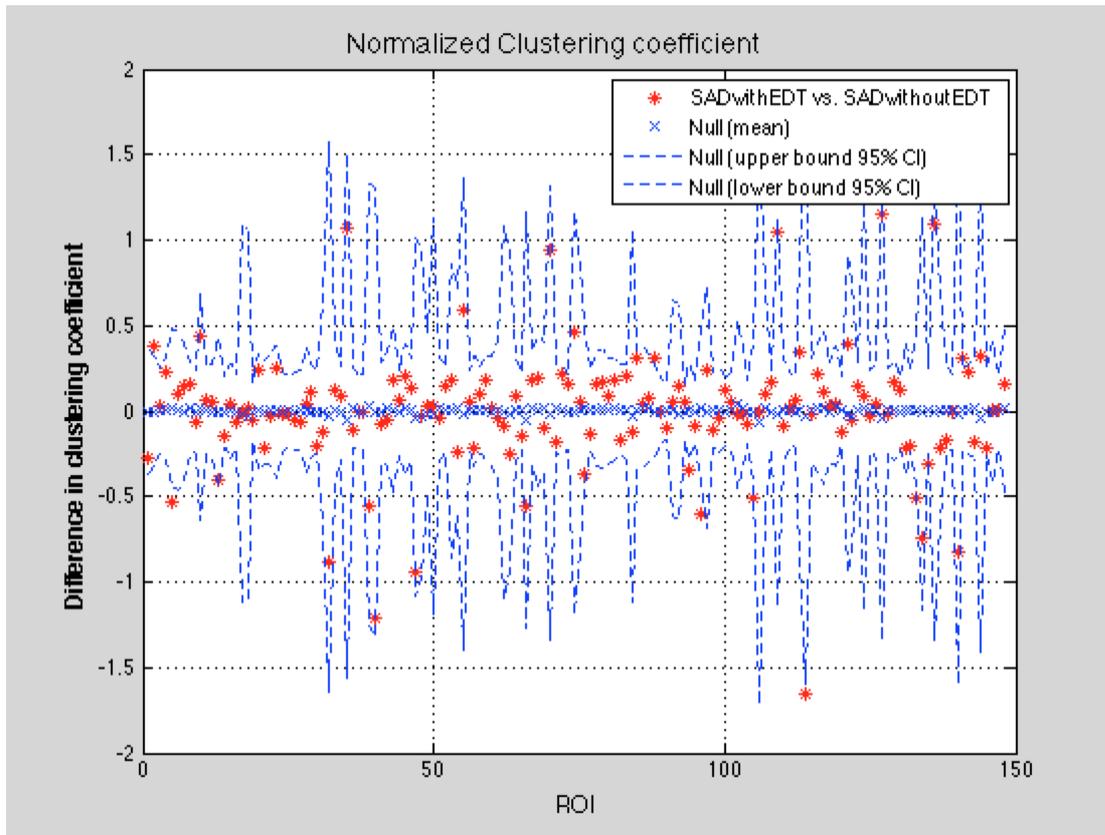
Figure 14 represents the clustering coefficients of all grey matter structures of the SAD with EDT and SAD without EDT groups. Significant level set at P < 0.05.

*7.6 Genotyping*

*7.6.1 Primary analysis of RGS2 and TPH2 genes*

The genotype counts and associated frequencies (total n = 56) for the polymorphisms in the tryptophan hydroxylase 2 (*TPH2*) and regulator of G-protein signaling (*RGS2*) genes were as follows: *TPH2* rs4570625 *G/G*: 42 (57.6%), *T/G*: 27 (37.0%), and *T/T*: 4 (5.5%); *RGS2* rs4606 *C/C*: 40 (54.8%), *G/C*: 22 (30.1%) and *G/G*: 11 (15.1%) (SeeTable 11 for more details). Genotype distributions for both polymorphisms were in accordance with those expected under Hardy-Weinberg equilibrium (p = 0.333 and p = 0.091 for *TPH* rs4570625 and *RGS2* rs4606, respectively).

Across-group genotype comparisons were undertaken. NHST regression analysis (logistic regression for categorical data and linear regression for continuous data) was selected to analyze differences in the sMRI left amygdala volume and ^{1}H-MRS neurometabolite data between *TPH2* and *RGS2* variants. The model was adjusted for age and gender. Neurometabolites and regional brain volumes that were found to be statistically different in previous sMRI and ^{1}H-MRS analysis of the groups with SAD only were included in the analysis. Total intracranial volume (ICV) was used as a covariate in the analysis of sMRI data.

Table 11

Presentation of allele groups in genotypes: RGS2 and TPH2

| Variant | Genotype/ Alleles | Count | Percentage | p-value |
|---------|-------------------|-------|------------|---------|
| *RGS2 rs4606* | *C/C* | 40 | 54.79 | p = 0.044 |
| | *G/C* | 22 | 30.14 | |
| | *G/G* | 11 | 15.07 | |
| *TPH2* | *G/G* | 42 | 57.53 | p = 1.00 |
| | *T/G* | 27 | 36.97 | |
| | *T/T* | 4 | 5.48 | |
| | *T* | 35 | 23.97 | |
| | *G* | 111 | 76.03 | |

*Note*. Table 11 represents the break down of each genotype in terms of the number of carriers of a specific allele group. It also represents the umber of carriers of specific nucleotides in the respective genotype base pairs.

### 7.6.2 Inferential statistics for RGS2 and TPH2

### 7.6.2.1    RGS2

For the effect of genotype on neurometabolite concentration, linear regression showed that there was a significant association between NAA and the *RGS2* genes when using the recessive model; individuals who were homozygous or heterozygous for the *C*-allele (i.e., with at least one *C*-allele [*CC* and *CG*]) (n = 41) had higher levels of NAA (mean = 5.94 (SE = 0.3605)) compared to *GG* homozygotes (n = 5) (mean = 2.44 (SE = 1.4919)) ($p < 0.001$; 95% CI for difference in mean values: -6.33 to -1.66). *RGS2* rs4606 genotype was also found to influence levels of Ins. Here again, individuals with at least one *C*-allele (n = 41) had higher levels of Ins (mean = 3.60 (SE = 0.2560)) compared to *GG* homozygotes (n = 5) (mean = 1.30 (SE = 0.7829)) ($p = 0.000134$; 95% CI for difference in mean values: -3.83 to -0.53). There were no significant associations between *RGS2* rs4606 and Glu ($p = 0.47$), Gln ($p = 0.54$) and PCr ($p = 0.31$). The small sample size must be taken into consideration with regards to these results.

### 7.6.2.2    TPH2

There were no significant associations between *TPH* genotypes and any of the neurometabolite measurements ($p > 0.05$ for all tests).

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# 8. GENERAL DISCUSSION

**The principle aim of this study was to explore mechanisms of SAD within the context of EDT. This section summarizes the major findings of each of the various study components, which included neuroimaging (^{1}H-MRS and sMRI), neurocognitive, and genetics assessments. It provides an integrated account of the neurobiological mechanisms in SAD within the context of EDT.**

8.1  *Overview of study findings*

The neurobiological mechanisms in SAD within the context of EDT have not been fully elucidated. This study undertook to determine whether individuals with SAD who also have EDT would demonstrate: (i) significantly greater reductions in grey matter volume in cortical and limbic system areas, (ii) significantly greater dysregulated neurometabolism within the amygdala, and (iii) more pronounced difficulties in neurocognitive test performance (e.g. verbal learning & memory, executive function and motor difficulties), than those without developmental trauma and relative to healthy controls. The study additionally attempted to address: (iv) whether the aforementioned neurocognitive impairments in individuals with SAD and EDT would correspond with region-specific sMRI volume shifts and dysregulated neurometabolism in the left amygdala. In addition (v) within the two SAD groups and the control group, the study investigated whether SAD with EDT participants were high-response carriers of variants of the *TPH2* and *RGS2* genes and whether they therefore showed enhanced amygdala reactivity, in terms of neurometabolite differences, relative to low-response variant carriers.  The first three hypotheses relate to putative pathophysiological mechanisms in SAD, while the last two deepen this understanding with regards to the interaction of genes and environment on observed brain effects.

The first three hypotheses have been addressed in previous chapters that mainly cover the comparative statistical findings on neurocognition, sMRI and ^{1}H-MRS between individuals with SAD with EDT, individuals with SAD without EDT and healthy controls. This paragraph briefly summarizes these findings, whilst the rest of the chapter integrates findings that shed light on pathogenic mechanisms in SAD in the context of EDT and therefore also addresses the last two hypotheses. First, the study found that the SAD with EDT group

demonstrated multiple verbal working memory difficulties, executive functioning difficulties, and poorer fine motor performance, compared to controls. Verbal memory difficulties were evident in both the SAD with EDT and SAD without EDT groups compared with controls. Second, verbal memory was the only cognitive test that was significantly different between the SAD without EDT group and controls. Our findings support previous findings of verbal memory difficulties in SAD. Third, the SAD with EDT group had significantly smaller left and right ACC and right thalamus grey matter volumes compared with controls. The volume of the left caudate was significantly smaller in SAD with EDT group compared with the SAD without EDT group. Fourth, a number of noteworthy neurometabolite differences were found in the left amygdala. There was evidence of lower NAA concentrations in both the SAD with EDT and in SAD without EDT groups compared with controls. Ins was also significantly elevated in the SAD with EDT group compared with controls. There was probable evidence for elevated Gln in the SAD without EDT group and evidence for elevated Glx in both the SAD with EDT and SAD without EDT groups.

8.2 *Neurocognition and sMRI brain morphometry in SAD with EDT*

Investigation of brain morphological abnormalities in anxiety disorders is key to understanding the neural correlates of these disorders (Maturana & Batrancourt, 2011), including SAD. In addition, morphological abnormalities of specific grey matter structures often correspond to functional activations of those same structures, as shown by fMRI (Gazzaniga, 2013; Maturana & Batrancourt, 2011). However, no studies have examined the relationship between neurocognitive functioning and structural brain abnormalities in SAD and SAD within the context of EDT (Sareen et al, 2007; Freitas-Ferrari et al, 2010).

Studies in SAD have focused predominantly on memory processing (Amir & Bomyea, 2010; Muckertz & Amir, 2014). Memory processing deficits are supported by several neuroimaging studies that implicate the temporal lobes, hippocampi, ACC and amygdala in SAD (Airaksinon, 2004; Freitas-Ferrari et al, 2010). Severe or chronic stress, including EDT, has detrimental effects on the structure and function of the hippocampus (Conrad, Lupien et al., 1999, Kirschbaum et al., 1996 and McEwen, 2000a; Finsterwald & Alberini, 2014), which has a key role in learning and memory. Trauma has been found to have long-lasting negative effects on verbal memory (Bremner et al., 1995; Johnsen & Asbjørnsen, 2008; Shin et al, 2015).  The findings of memory difficulties in both SAD groups is also supported by a number of studies that point to information processing biases which have been suggested to

play a causal role in the pathophysiology of SAD (Amir & Bomyea, 2010; Muckertz & Amir, 2014). Majer et al examined 47 healthy adults, who formed part of a larger study conducted in the Unites States using the Cambridge Neuropsychological Test Automated Battery (CANTAB) in combination with the Wide-Range-Achievement-Test (WRAT-3) (Majer et al., 2010), and the Childhood Trauma Questionnaire (CTQ). In assessing cognitive function in relation to the type and severity of EDT, the authors found that physical neglect and emotional abuse were associated with difficulties in memory functioning (Majer et al., 2010). EDT may, therefore, explain some of the neurocognitive deficits in SAD.

8.2.1 *Memory and sMRI brain morphometry in SAD with EDT*
Both the SAD with EDT and the SAD without EDT groups' demonstrated difficulties in immediate and delayed memory recall compared with controls.  However, statistically significant correlations between memory tests and brain volumes were only found in the SAD without EDT group. There was a correlation between immediate memory recall (r = .503, p =. 020), as measured by the WMS, and the left ACC in the SAD without EDT group. As such, smaller left ACC volume was associated with poorer immediate and delayed memory recall performance. There was also a significant correlation between delayed memory recall, as measured by the HVLT (r = .456, p = .033) and the right ACC; such that smaller right ACC volume was associated with poorer delayed memory performance in the SAD without EDT group. There may be reduced capacity for the ACC to exercise inhibitory control over limbic circuitry in individuals with SAD, specifically over the amygdala. For example, in a study that examined the effects of paroxetine in drug naïve SAD patients the hopactivation in the ACC and thalami was found (Giménez, et al., 2013).

A number of studies have failed to document neurocognitive deficits in individuals with SAD. For example Castaneda, et al. (2011) found no significant differences in neurocognitive tests of executive functioning, memory or fine motor performance in individuals with SAD compared with controls. Other studies also do not support neurocognitive deficits in SAD. A recent study examined a series of 9 neurocognitive assessment instruments in 25 individuals with generalized SAD (Sutterby & Bedwell, 2012) and found no significant neurocognitive difficulties or deficits compared to healthy controls. The authors concluded that neurocognitive difficulties are unlikely to account for the information processing biases in SAD (Sutterby & Bedwell, 2012). Coles and Heimberg (2002) found no significant memory biases, specifically for threat relevant information, in SAD compared with other anxiety

disorders (panic disorder, posttraumatic stress disorder and obsessive compulsive disorder). Of relevance to this study is the relationship between EDT and later life cognitive functioning, where a significant relationship has been found to exist (Majer et al., 2010). Stressful and aversive experiences such as EDT may ultimately lead to deficits and difficulties in cognitive functioning in individuals with SAD. It was previously mentioned that individuals with SAD report significantly higher rates of EDT compared to healthy controls.

8.2.2 *Executive functioning and sMRI brain morphometry in SAD with EDT*
A relationship between amygdala size and executive function has been shown. Damage to the amygdala or amygdala dysfunction can directly affect executive functioning (Brand, et al, 2007), although the current evidence base is sparse in this regard (Brand et al, 2007). Brand et al. (2007) found that patients with selective amygdala damage had lower scores in both decisions under ambiguity and decisions under risk. Both type of decision making capacity are demonstrative of executive functioning difficulties. In the present study, we found a significant correlation in the SAD with EDT group between WCST non-perseverative errors and right amygdala volume (r = -.427, p = 0.042); in other words increased performance on the WCST corresponded with smaller right amygdala volume.  Non-perseverative errors represent executive function difficulty (Lezak et al, 2012; Nyhus & Barceló, 2009) involving the prefrontal cortex (Nyhus & Barceló, 2009). The prefrontal cortex is thought to be involved in top-down regulation of the amygdala, while the amygdala in turn modulates prefrontal cortical activity (Oh, 2012; Motzkin et al, 2015; Eden et al, 2015). Poor performance on the WCST in the SAD with EDT group compared to controls and the observed relationship between WCST non-perseverative errors and amygdala volume may be indicative of the relationship between fronto-amygala circuitry and executive functioning in SAD with EDT. However, only the correlation of the WCST with right amygdala volume and not left amygdala volume was significant.

It remains unclear as to the functional role that EDT plays in the pathophysiology of the specific structural differences observed in the SAD with EDT group. EDT compromises a number of vulnerable brain regions which include the hypothalamic–pituitary–adrenal axis, the amygdala, the hippocampus, and prefrontal cortex, which are linked to dysfunctional neurocognitive processes (throughout adult life) (Wilson et al, 2011). EDT is known to alter neural development and to affect on limbic system functioning (Matz, Junghöfer Weber,

Wienbruch & Rockstroh, 2010) and possibly even the amygdala (Schmahl et al, 2003; Rogers et al, 2009). Clarifying the precise nature of amygdala-prefrontal interaction in trauma-related disorders is essential for understanding the pathophysiology of these disorders and for identifying optimal treatment targets (Oh, 2012; Arnsten et al, 2015). Pathophysiological processing of the amygdala may affect executive functioning by affecting bottom-up processing of the prefrontal cortex (Oh, 2012; Motzkin et al, 2015). EDT may have a role to play in SAD pathophysiology. The SAD without EDT group displayed no significant differences in executive functioning compared with SAD with EDT and control groups. The amygdala is particularly susceptible to EDT and EDT may affect amygdala-frontal network processing and connectivity (Pechtel & Pizzagalli, 2011; Oh, 2012). There is accumulating evidence that the amygdala and the prefrontal cortex play critical roles in conditioning and the extinction of traumatic fear memories. The prefrontal cortex regulates stress-induced fear and anxiety-like behaviour via inhibitory effects on amygdala output and processing (Akirav and Maroun, 2007, Bishop, 2007; Oh, 2012). Functionally, the amygdala is an essential component of the circuit involved in implicit emotional learning and memory, emotional modulation of memory, emotional influences on attention and perception, emotional and social behaviour, and emotional inhibition and regulation. Many of the aforementioned processes relate specifically to executive functioning (Phelps & LeDoux, 2005). In particular, the amygdala mediates the acquisition and expression of conditioned fear and the enhancement of emotional memory (Koenigs and Grafman, 2009).

8.2.3 *Fine motor functioning and sMRI brain morphometry in SAD with EDT*
This study found a significant correlation between fine motor performance in the dominant hand and left caudate nucleus volume in the SAD with EDT group (r = -0.434, p = 0.039), with smaller left caudate volume corresponding with slower fine motor performance. There were no significant correlations with non-dominant fine motor performance and structural grey matter volumes (in the amygdala, caudate nucleus and ACC) in the SAD with EDT group. The caudate nucleus is a basal ganglia structure involved in fine motor function and dexterity (Tonkonogy & Puente, 2009). The caudate nucleus relays information to the thalamus about voluntary and conscious planning in movement (Grahn et al, 2009). It is also a structure that plays a role in fear processing (Tonkonogy & Puente, 2009). There is compelling evidence to suggest that the limbic system has a role in motor performance (Balaban, 2002). The limbic system may have a compensatory role in processes involved in modifying motor control in response to threat and fear, where a trade-off between increased

vigilance in threatening situations and reduced motor control exists (Balaban et al, 2002). The caudate nucleus may be especially important in this process and may contribute to Balaban's (2002) original model of understanding motor response processes when responding to fearful situations or when anxious. The reduced caudate nucleus volumes may be associated with fine motor functioning differences observed in the SAD with EDT group. Impairments in psychomotor speed have been reported in the EDT literature, where slower response times in individuals with developmental traumatization have been observed (Palmer et al., 1997).

8.3 *Amygdala neurometabolite dysregulation and morphometry in SAD within the context of EDT*

The amygdala was the principle focus of this study. There were no statistically significant differences in amygdala volume among the three groups in this study. This is in keeping with other studies that have found no differences in amygdala volume in individuals with SAD compared with controls (Syal et al, 2012; van Tol & van der Wee, 2010). However, a few studies have documented volumetric differences in the amygdala in adults (Irle et al, 2010) and adolescents (Muller et al, 2013) with social fears, compared with healthy individuals. Volume differences (predominantly reduced volume) have generally only been observed in the right amygdala in individuals with SAD, and not in the left amygdala, compared with healthy controls (Meng et al., 2013), which was the site of measurement of neurometabolites in this study.

Firstly, both SAD groups demonstrated a larger Glu/Gln signal compared with the control group, in the left amygdala, approximating similar findings in other research of raised Glu/Gln signal in limbic regions, compared with controls (Phan et al, 2005; Pollack et al, 2008). The limbic system has been a focus of investigation in anxiety disorders and SAD especially with regards to glutamatergic neurometabolism (Bermudo-Soriano et al, 2012; Harvey & Shahid, 2012). Given that there were no differences in Glu and Glx between the SAD groups, it may be assumed that EDT per se does not account for differences Gln and Glx neurometabolism between SAD and controls.

Secondly, differences in NAA were observed in both the SAD groups compared with controls. However there was no difference between the SAD with EDT and the SAD without EDT groups. Regional reductions in NAA have been argued to represent an in vivo signal of compromised neuronal integrity or fitness (Barker, 2001; Bertolino et al, 1997, 2002). Some

209

in vivo studies have shown a corresponding lower NAA and higher Ins levels in disorders where the pathology involves neuronal loss and/or gliosis (Bitsch et al, 1999; Kantarci et al, 2004; Coupland et al, 2005). Ins and NAA are both neurometabolites involved in cellular integrity. The SAD with EDT group displayed a greater mean reduction in NAA than the SAD without EDT group, although this was not statistically different. Some studies have shown a reduction in NAA and related neuronal density and functional integrity due to early life stress (Coplan et al, 2010; Mathew et al, 2003).

Ins plays a substantial role in glial metabolism and reductions in Ins are likely to indicate glial loss or altered glial metabolism (Coupland, 2005). Lower levels of Ins were found in the SAD with EDT group compared with controls and SAD without EDT group. EDT may play a mediating role or be associated with Ins dysregulation, as there were no differences found in the SAD without EDT group compared to controls. Dysregulated levels of Ins have been associated with astroglial deficits due to early life stress (Zhang et al., 2013). Lastly there was a lower concentration in PCr in the SAD with EDT group compared with controls. PCr is a principle neurometabolite involved in cellular energy utilization (Andres, et al, 2008). Lower PCr signal may, therefore, represent a lower output or dysregulated neurometabolism of the left amygdala in individuals with SAD with EDT (Tupler et al., 1997; Shioiri et al 1996).

Amygdala activation can be accurately measured by the degree of metabolic activity and through measurement of neurometabolites using ^{1}H-MRS. There have been very few ^{1}H-MRS studies in SAD (Freitas-Ferrari et al, 2010), but of those that have been performed, our findings closely approximate other studies. For example, our findings of higher concentrations of Glx, PCr and Ins approximate those of an earlier ^{1}H-MRS study conducted by Tupler et al. (1997) who conducted a Clonazepam treatment study of 19 individuals with SAD matched with 10 controls. Increased concentrations of NAA, Ins, Glu and Cr in subcortical grey matter in the ACC were found compared with controls (Tupler et al, 1997). In sum, several of the abovementioned findings point to dysregulated amygdala metabolism in the SAD with EDT group. Neurometabolite abnormalities together with preserved amygdala volume are suggestive of lower metabolic output indicated by increased Glu/Glx and lower NAA and Ins. These neurometabolite abnormalities suggest functional dysregulations in the cellular processes of the amygdala rather than morphological changes.

8.4 *RGS2 and TPH2 genes and amygdala morphometry and neurometabolism in participants with SAD with EDT*

Although the study predominantly examined the role of EDT in the pathogenicity of SAD via neurocognitive and neuroimaging mechanisms, a secondary objective was to examine gene variants associated with amygdala function in SAD.  The two polymorphisms under investigation were RGS2 rs4606 (Smoller et al, 2008) and TPH2 rs4570625. This was examined in a small sample and as such findings need to be cautiously interpreted.

There was no significant association of RGS2 polymorphic variants and social anxiety severity, however heterozygous (TG) carriers of the TPH2 gene had higher total social anxiety scores and higher scores on the social fear and avoidance subscales than homozygous (GG) carriers. We found no association between RGS2 rs4606 and TPH2 rs4570625 and amygdala volumes. These findings are interesting considering that smaller amygdala volumes have been documented in homozygous TPH2 carriers (Inoue et al., 2010) and the rs4570625 variant of TPH2 has also been found to correspond with greater functional activity of the amygdala (Furmark et al, 2008; Furmark et al, 2009). In addition *RGS2* explained approximately 10% to 15% of the variance in amygdala and insular cortex activation to emotional faces (Smoller et al. 2008). *RGS2* is also known to play a role in increased limbic system activation, especially in the amygdala and insula (Hirshfeld-becker, Micco, Wang & Henin, 2014). Oxytocin which may be released through positive social experience is known to increase activation of the *RGS2* gene which is responsible for the production of the regulator of G-protein signaling in the central nucleus of the amygdala (Kagan, 2014).

TPH2 is the rate-limiting enzyme in the synthesis of neuronal 5-HT and thus plays a key role in regulating 5-HT neurotransmission in the amygdala and limbic system (Walther & Bader, 2003; Zhang, Beaulieu, Sotnikova, Gainetdinov, & Caron, 2004). The first examined rate-limiting enzyme in 5-HT synthesis was tryptophan hydroxylase (TPH1). It had initially been thought that TPH1 was derived from a single gene until a second TPH isoform (TPH2) was described (Cote et al., 2003; Walther and Bader, 2003; Walther et al., 2003; Inou et al, 2010). While TPH1 is primarily expressed in the peripheral nervous system, TPH2 is predominantly expressed in the brain and exclusively maintains brain 5-HT synthesis across the lifespan (Cote et al., 2003; Gutknecht et al., 2007; 2009; Walther et al., 2003; Zhang et al., 2004; Zill et al., 2004a).

We found that individuals who carried at least one C allele of the *RGS2* gene, compared with homozygous *GG* carriers, had higher levels of NAA and Ins; both metabolites are indicative of neuronal integrity (Zhang et al 2013). Dysregulated neurometabolism may imply reduced functional processing, therefore specific metabolic dysregulation in the amygdala in C variant carriers of the *RGS2* genotype may also imply reduced functional activity of the amygdala in this group. Differences in Ins concentration between *RGS2* CC/CG and G/G carriers may be a pathophysiological marker of SAD. Our findings may provide more specificity to findings of Smoller et al (2008), who found markers spanning *RGS2* were associated with childhood behavioral inhibition, a temperamental precursor of SAD. However, Smoller et al. (2008) also documented, in independent samples, an association between *RGS2* rs4606 (previously associated with *RGS2* expression), introversion (a core personality trait in SAD) and increased limbic activation in the insular cortex and amygdala, during emotional processing (Smoller, et al, 2008).

Ins regulates glial cell functional integrity of which astrocytes being some of the most abundant. Astrocytes are the major cellular constituents of the central nervous system (CNS) and occupy 20–30% of brain volume (Kim, et al., 2006). *RGS2* expression is also involved in astrocyte regulation through reciprocal feedback of adrenergic receptor-mediated signaling (induced by isoproterenol) (Kim et al., 2006; Ota et al., 2013). The findings of this study suggest that Ins plays a role in astrocyte regulation through *RGS2* expression in the amygdala. This finding is interesting, given that Ins is a principle neurometabolite involved in the functional integrity of glial tissue and *RGS2* expression has a principle role in glial cellular function. In addition Ins trisphosphate, a mediating hormone involved in signal transduction and lipid signaling related to Ins metabolism, is involved in mediating the isoproterenol-induced up-regulation of *RGS2* mRNA (Kim et al., 2006). Of note, the SAD with EDT group demonstrated reduced Ins in the amygdala.

In sum, the *RGS2* genotype has also been associated with stress symptoms following experiences of trauma throughout life (Amstatder et al., 2009). There were no differences between the SAD groups in *RGS2*, however *RGS2* may play a mediating role in SAD pathophysiology through dysregulating glial cell metabolism. Thus, there may be a complex interplay of gene and environment on amygdala dysregulation.

8.5 *Mechanisms in SAD in the context of EDT – An integration and summary of findings*

In summary this study examined grey matter volume differences – principally of limbic and related circuitry; neurometabolites of the left amygdala; neurocognitive dysfunctions; and differences in structural brain volumes between *TPH2* and *RGS2* genotypes, in individuals with SAD within the context of EDT, individuals with SAD without EDT and healthy controls. The mechanisms investigated in this study represent pathogenic disturbances that may underpin SAD in individuals with early life trauma.

8.5.1 *The role of traumatisation in SAD pathophysiology*

EDT plays a specific role in SAD, characterized by greater attenuation of the amygdala and related neural structures and more cognitive dysfunction. EDT does not appear to affect the clinical presentation of SAD in terms of level of reported social anxiety, avoidance behavior, or associated comorbidity (such as major depression or generalized anxiety disorder), but rather affects the neurocognitive performance of individuals presenting with SAD with and without EDT.

We hypothesized that individuals with SAD within the context of EDT would have morphological grey matter abnormalities that would approximate individuals with PTSD. However the finding of no amygdala volume differences in the SAD with EDT group compared with controls and the SAD without EDT group were generally not in keeping with findings from other studies of reduced amygdala volume in individuals who have experienced traumatic stress (Weber et al., 2013; Rogers et al., 2009). Further, we did not find hippocampal volume reduction, which has been a consistent finding in the PTSD and EDT literature (Smith, 2005; Litizia et al., 2008; Shu et al., 2013). Hippocampal volume reduction is a key morphological indicator of the enduring effects of traumatic experience on the brain (Shu, et al., 2013; Liu et al., 2011). However the reduced volume of the ACC approximates other studies of reduced ACC volumes found in PTSD (Hamner et al, 1999; Yamasue, et al, 2003; Woodward et al, 2006; Kitayama et al, 2005; Corbo et al, 2005; Rogers et al, 2009). Dysregulated neurometabolism has also been observed in the ACC in PTSD occurring both in adulthood and childhood (De Bellis et al 2000; Rosso et al, 2013; Schuff et all, 2008). There is also high functional connectivity between the ACC and amygdala (Weber et al, 2013). The ACC is known to mediate motor responses in emotional reactions involving fear and anxiety (Tonkonogy & Puente, 2009). This finding of reduced ACC volume in SAD within the context of EDT requires further investigation.

Structural brain abnormalities in the ACC and caudate nucleus in relation to EDT have been found in other research. A study assessing childhood aversive events, ACC, hippocampus, amygdala and caudate nucleus volumes in 265 healthy Australian men and women (Cohen et al., 1996) found that individuals who reported more than two aversive events had significantly smaller ACC and caudate nucleus volumes compared to individuals with less aversive experiences. These effects were not found for hippocampal or amygdala volumes (Cohen et al., 1996). This appears to be in keeping with findings of this study. There is good evidence that neural networks involving the amygdala and the ACC are interconnected in the processing of social fear related information (Brühl et al., 2014). It is known that exposure to EDT or early life stress is sufficient to cause significant structural remodeling of the principle projection neurons within the rodent prefrontal cortex (de Kloet et al., 2005; McEwen & Milner, 2007; Holmes & Wellman, 2009; Sapolsky, 2003). In parallel, there is growing evidence that stress induced alterations in prefrontal cortical neuronal morphology are associated with deficits in rodent executive functions such as working memory, attentional set-shifting and cognitive flexibility, as well as emotional dysregulation in the form of impaired fear extinction (Holmes & Wellman, 2009).

Traumatic stress has been suggested to affect the production of long-term memory by producing effects on memory consolidation through basolateral amygdala activation (Chavez et al, 2013) and stress pathways of the hippocampal-pituitary-adrenal axis (Finsterwald & Alberini, 2014). Chronic stress mostly impairs hippocampal-dependent mechanisms and functions and not amygdala related memory processing (Finsterwald & Alberini, 2014). Stressors and traumas may negatively impact on cognitive tasks and related memory functions that are more complex (e.g. verbal memory processing) and may improve simpler cognitive tasks, such as spatial memory processing (Finsterwald & Alberini, 2014). Our findings suggest SAD with EDT may be associated with more pervasive neurocognitive difficulties which requires closer examination in studies of SAD by examining the long-term impact of EDT (Sutterby & Bedwell, 2012; O'Toole & Pederson, 2011).

8.5.2 *Neural circuitry findings in SAD within the context of EDT*
Miskovic & Schmidt (2012) suggest a need to adopt an integrative, network-based approach to the study of the neural substrates underlying SAD. Qui et al (2011) in a study of resting state networks and the default mode network suggest that more diffuse neurocircuits are

involved. In a recent meta- analysis and review of neuroimaging in SAD, Brühl et al. (2014) examined a number of functional and structural neuroimaging studies (n= 36) and found common architectural and functional relations between subcortical and cortical brain circuits. Of specific relevance was the reduced connectivity between limbic, frontal (e.g. ACC) and parietal regions (Brühl et al., 2014). Thus, network-based approaches to understanding SAD pathophysiology rather than examining nodal or region specific functioning have been recommended. Although the findings of this study do not present information on functional neural network relationships, the amygdala-metabolic dysregulations together with specific structural changes in associated neurocircuitry are suggestive of specific circuitry contributions in individuals with SAD who have experienced EDT.

It is important to note that studies have found functional correlates in the ACC, thalamus, caudate nucleus and amygdala (Giménez et al. (2014). Further, the pharmacologic effects of anxiolytic agents can be mapped as functional changes in fear, stress and anxiety brain circuits (Giménez et al. 2014). For example, administration of the antidepressant paroxetin, in patients with SAD (n=17) compared with placebo administration (n=16), reduced the activation of the insula, thalamus and ACC. Further resting-state fMRI assessment using Independent Component Analysis indicated that paroxetine reduced functional connectivity in the insula, thalamus and ACC when compared with a placebo. Conversely, paroxetine compared to placebo produced activation of the right amygdala and bilateral insula but had no effects in the ACC (Giménez, et al., 2014). The findings of these studies confirm architectural and functional connectivity between the thalamus, ACC and caudate nucleus, as well as the amygdala in the pathophysiology of SAD (Giménez et al. 2014).

The ACC has been considered to be an important component in the fear and anxiety circuitry and plays a crucial role with a number of interconnected brain regions such as the orbitofrontal cortex, prefrontal cortex and a number of subcortical limbic structures (e.g., the nucleus accumbens and amygdala) (Ressler & Mayberg, 2007; Aupperle & Paulus, 2010). Various subcomponent structures of the ACC also interconnect with areas such as the ventral striatum and insula, both being important components in reward behaviors and interoceptive regulation (Aupperle & Paulus, 2010).

Giminez et al. (2012) found a statistically significant increase in in SAD participants in task-induced functional connectivity between the ACC and scrutiny-perception-related regions,

such as the amygdala. The distinct brain volume abnormalities in the SAD with EDT group suggest some specificity of pathophysiology in SAD with EDT. Graph analysis, a statistical technique used to further explore relationships between various grey matter structures in the brain demonstrated a significant difference in the ACC between the SAD with EDT compared with the SAD without EDT, with smaller ACC volumes in the SAD with EDT group. Blair et al (2012) found reduced ACC activity in emotional attentional control in SAD.  In their study, Blair et al (2012) examined participants with SAD and found reduced activity in the ACC. Controls showed significantly increased activity during emotion regulation relative to emotion-picture viewing. This was in contrast to SAD participants who showed no such increases. This was interpreted in the study to suggest reduced activation of the ACC in emotion regulation compared to controls. A reduced ability to recruit regions implicated in top-down attention might represent a general risk factor for anxiety disorders (Blair et al., 2012). Our findings of reduced right and left ACC volume in the SAD with EDT group appear to support the findings of Blair (2012), with volume reduction suggestive of a structural abnormality of the ACC, which is important in modulating social anxiety and social threat.

Hahn et al (2011) suggest that there is reciprocal involvement of amygdala hyperactivation with the ACC and thalamus during social stress.  Patients with SAD suffer from excessive anxious responses in social interactions, leading to avoidance behavior and social impairment. Although the amygdala has a central role in perception and the processing of threatening cues, there is little known about the involved networks and corresponding network architectures in SAD (Hahn, et al., 2011). The study by Hahn et al. (2011) mainly investigated the functional connectivity network of the amygdala in participants with SAD to identify regions that might influence amygdala reactivity via modulatory pathways. They examined 10 participants with anxiety disorders (social and/or panic) and 27 healthy controls who underwent a facial emotion processing task as well as a 6-minute resting state fMRI. Individual voxel-wise functional connectivity maps were calculated using the amygdala as a seed region. Group comparisons were done by random-effects analysis in SPM. Participants with SAD exhibited amygdala hyperactivation during the emotional task and decreased functional coupling of the left amygdala with the medial orbitofrontal cortex and the posterior cingulate cortex. Further, exploratory analysis revealed reduced connectivity and a marked functional separation between the medial orbitofrontal and ACC in the SAD group. Their results suggest alterations in amygdala connectivity networks with the ACC in SAD (Hahn et

al, 2011). Combined with amygdala hyperactivation, their findings further corroborate proposed dysfunction of fronto-amygdala inhibition in anxiety disorders and indicate a modulatory influence of the ACC on threat perception and processing (Hahn et al, 2011).

Further Perlman and Pelphrey (2013) investigated brain mechanisms involved in the development of affective regulation in 5- to 11- year-olds and an adult comparison sample using fMRI. Children and adults displayed differing patterns of increased ACC and decreased amygdala activation during episodes in which emotion regulation was required. They found high functional connectivity between the amygdala and ACC in emotion regulation and in functions related to the executive control of emotions (Perlman & Pelphrey, 2013). There may be structural as well as functional connectivity processes between the ACC and amygdala in SAD pathophysiology. Further the observed smaller ACC volumes in the SAD with EDT group compared with the SAD without EDT group are an important observation in amygdala-frontal network pathology in SAD within the context of EDT.  Executive functioning difficulties may follow from this, however this was non-supported directly in this study.

The pathophysiology of SAD with EDT appears to involve diffuse neurocircuitry with accompanying neurocognitive performance difficulies in fine motor performance, verbal memory and executive function. The neurocircuitry differences together with the neurocognitive difficulties in the SAD with EDT group compared to controls suggest cortical-subcortical networks (Tonkonogy & Puente, 2009). This requires broadening the scope of investigation beyond examination of amygdala functioning and neurometabolism. Examination of cortical processes, including functional and neurometabolite assessments, such as in the ACC, may be equally important in the pathophysiology of SAD. This appears especially important when considering the effects of early life trauma. In summary there were greater differences in amygdala functioning in terms of dysregulated neurometabolism in the SAD within the context of EDT group compared with controls, even though there were no volume differences of the amygdala across the three study groups. The study found only one difference between the SAD groups in neurometabolism (only Ins was different between the groups) therefore making it difficult to establish the role EDT plays in the pathophysiology of SAD. Further research of these mechanisms and findings is warranted. The mechanisms discussed in this study are unlikely to account for all the processes involved in the pathophysiology of SAD within the context of EDT. Distinguishing the precise mechanisms

underlying the pathophysiology of SAD within the context of EDT is clearly a complex task.

8.6 *Principle study findings*

(i.) Decreased grey matter volume differences were found in the left thalamus and the right and left ACC in individuals with SAD with EDT compared with controls and decreased grey matter volume wasfound in the left caudate nucleus in the SAD with EDT group compared with controls.   ..

(ii.) The right and left ACC was smaller in individuals with SAD and EDT compared with controls. Therefore the ACC represents an important component in the pathophysiology of SAD within the context of EDT.

(iii.) Both SAD without EDT and SAD with EDT groups demonstrated neurometabolite dysregulation compared with controls; similar to what has been found in the ACC and other cortical and sub-cortical grey matter structures in the SAD literature.

(iv.) There was greater neurometabolic dysregulation in the left amygdala in SAD in the context of EDT, as demonstrated by dysregulated Glx, NAA and Ins compared with controls.

(v.) More diverse neurocognitive impairments (e.g. in verbal memory, executive functioning and motor functioning) were found in the SAD with EDT group compared with controls.  Where more discrete verbal memory difficulties were found in individuals with SAD without EDT compared with controls.

(vi.) RGS2 may play a role in amygdala neurometabolism, however this may not be specific to the pathophysiology of SAD.

(vii.) It is important to note that there were few differences on neurocognitive, neurometabolite and brain morphometry data between the SAD with EDT and SAD without EDT grousp. The only significant difference between the two groups eas in one area of brain morphometry, the left caudate nucleus.

8.7  *Contribution to knowledge gaps*

(i)     The pathophysiological processes in SAD may be influenced by environmental determinants. Better understanding the contribution of EDT in SAD may help to delineate pathogenic mechanisms. This is, to our knowledge, is the first study to compare neurophysiological and neurocognitive parameters in SAD in the context of EDT.

(ii)    Findings of neurocognitive difficulties in childhood trauma have been inconsistent. This is the first study to assess neurocognitive functioning in a sample of South African individuals with SAD and a history of childhood adversities. The study provides evidence for multiple neurocognitive difficulties in individuals with SAD with early life stress.

(iii)   This is also the first study to explore the relationship between neurocognition and sMRI brain volumes in SAD within the context of EDT.

(iv)    There are also no studies that have examined neurometabolites in the amygdala in SAD. This study provides evidence for neurometabolite dysregulation in the left amygdala in SAD and in SAD in the context of EDT. Future research may elaborate on these findings and build on the methodologies used in this study to examine neurometabolite dysregulation in SAD and SAD in the context of EDT.

(v)     The study used a combination of null hypothesis significance testing and Bayesian statistical analysis. This allowed for a more robust analysis of the data, given sample size limitations and lack of prior information on many of the hypotheses under investigation in this study. Bayesian analysis also provides a different approach, philosophically towards understanding the mechanisms of SAD within the context EDT.

8.8   *Limitations*

(i)      Several limitations are worth noting. Firstly the sample was drawn from private practices, radio advertising and online media and advertising, which may limit the kinds of demographic profiles of individuals recruited into the study. Most of the research participants where white, which is not representative of all ethnic diversity of SAD. This affects the generalizability of findings to other ethnic groups.

(ii)      The CTQ-SF is a retrospective measure of childhood trauma. Retrospective measurements based on past events often involve some level of recall bias.

(iii)      The statistical analyses involving GLM, are likely to be affected by the small sample size in conjunction with the large number of multiple comparisons and predictor variables.  This increases the risk of making statistical errors (i.e. Type I errors). MCMC helps to balance these errors. A Bayesian approach treats power differently to NHST and considers the evidence from the position of beliefs of probability rather than from a position of confirming or rejecting a hypothesis.

(iv)      The amygdala is a particularly difficult area of the brain to scan using ^{1}H-MRS (Nacewicz et al, 2012) making the average scan time very long and also significantly affecting the overall success rate of scans.

(v)      For many participants, we were not able to ascertain the temporal onset of EDT in relation to SAD.

(vi)      The study was specifically a cross sectional examination of three groups in comaring differences with SAD within the context of EDT. The study was unable to show many differences between the SAD groups themselves. Further research is therefore recommended, such as a longitudnala design, which may be able to examine these mechanisms more thoroughly.

(vii)      Lastly functional magnetic resonance imaging (fMRI) data of the amygdala, which may have provided additional insights, were not incorporated into these analyses.

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225

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# 9. CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

*9.1 Conclusion*

The study provides novel findings in SAD within the context of EDT. In conclusion the study demonstrates differences in pathogenic mechanisms in SAD with EDT compared with SAD without EDT and healthy controls. Of these it appears that there are a number of regional brain volume differences in individuals with SAD and EDT compared with controls. There were more differences between the SAD with EDT group compared to controls than the SAD without EDT group when compared with controls (*See* **Appendix F**, *for further details on the comparisons between the SAD group findings from pairwise statistical analysis). There were however few differences between the SAD groups except left caudate nucleus volume*.  These findings demonstrate the specificity of the pathophysiology of SAD within the context, even though there were no differences in the presentation of social anxiety symptoms and comorbidity between the two SAD groups. However the study was unable to clearly demonstrate the differences in the pathophysiological mechanisms between individuals with SAD with EDT and individuals with SAD alone. The study also highlights the involvement of other brain regions in the pathophysiology of SAD within the context of EDT, such as the ACC.

Additionally individuals with SAD and EDT displayed a greater number neurocognitive dysfunctions, spanning a number of neurocognitive domains, than individuals with SAD without EDT. Both groups displayed memory difficulties, however EDT appears to contribute to fine motor performance and executive functioning difficulties in individuals with SAD with EDT. However the role that EDT plays in the neurocognitive dysfunction in SAD warrants further investigation.

Lastly the amygdala is a central component in the pathophysiology of SAD and has been widely investigated in emotional and social fear related brain processes. Individuals with SAD with EDT had greater amygdala neurometabolite dysregulation compared to controls. Both groups demonstrate dysregulated Glx, which is in keeping with the current literature, however individuals with SAD with EDT also demonstrated dysregulated NAA, Ins and PCr.

These neurometabolites generally describe the functional integrity of the neural structures in question.

## 9.2  *Directions for future practice and research*

EDT may inform future treatment regimes, as SAD with EDT appears to represent a different set of neurobiological mechanisms at play than SAD without EDT. This may influence the effects of various psychopharmacological treatment regimes and approaches as well as the effects of behavior and cognitive behavior therapy. In the psychotherapy of SAD in the context of EDT, information processing difficulties such as planning and abstraction are important considerations. This study investigated two genotypes, however a significant shortcoming was statistical power due to the small sample size.

## *9.3 Recommendations for future research*

The contribution of EDT to the pathophysiology of SAD is poorly understood. We recommend extending this research to larger samples and to investigation of other mechanisms of SAD within the context of EDT. It is also pertinent to conduct functional imaging studies to complement the structural and ^{1}H-MRS findings of this study. We also recommend that future studies employ a Bayesian statistical framework to build on the knowledge produced by this research. A Bayesian framework will allow future researchers to build on the knowledge and beliefs, in the form of statistical priors, about what is known in this study about the mechanism of SAD within the context of EDT.

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**Appendix A**

**Bayesian analyses for neurocognitive data**

**Figure A(i)**
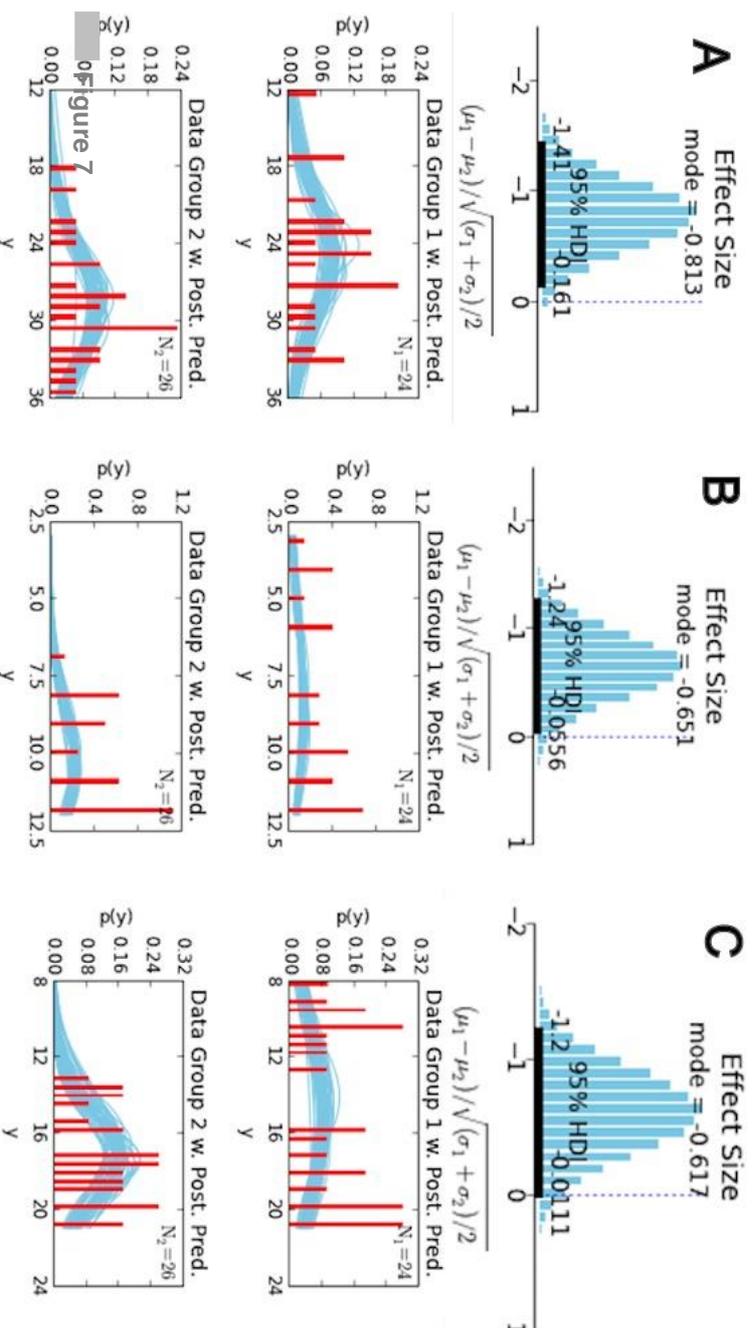


Appendix A Figure A(i) Indicates the BEST with MCMC simulations of significant differences in the verbal memory tests for the SAD without EDT group and controls, indicating: *A* Hopkins verbal learning immediate recall test (*BEST Effect Size -0.813, 95% HDI [-0.161, -1.41]*); *B* Hopkins verbal learning delayed recall test (*BEST Effect Size -0.651, 95% HDI [-0.0651, -1.24]*); *and C* Weschler memory scales logical memory immediate recall (*BEST Effect Size -0.617, 95% HDI [-0.0111, -1.2]*).
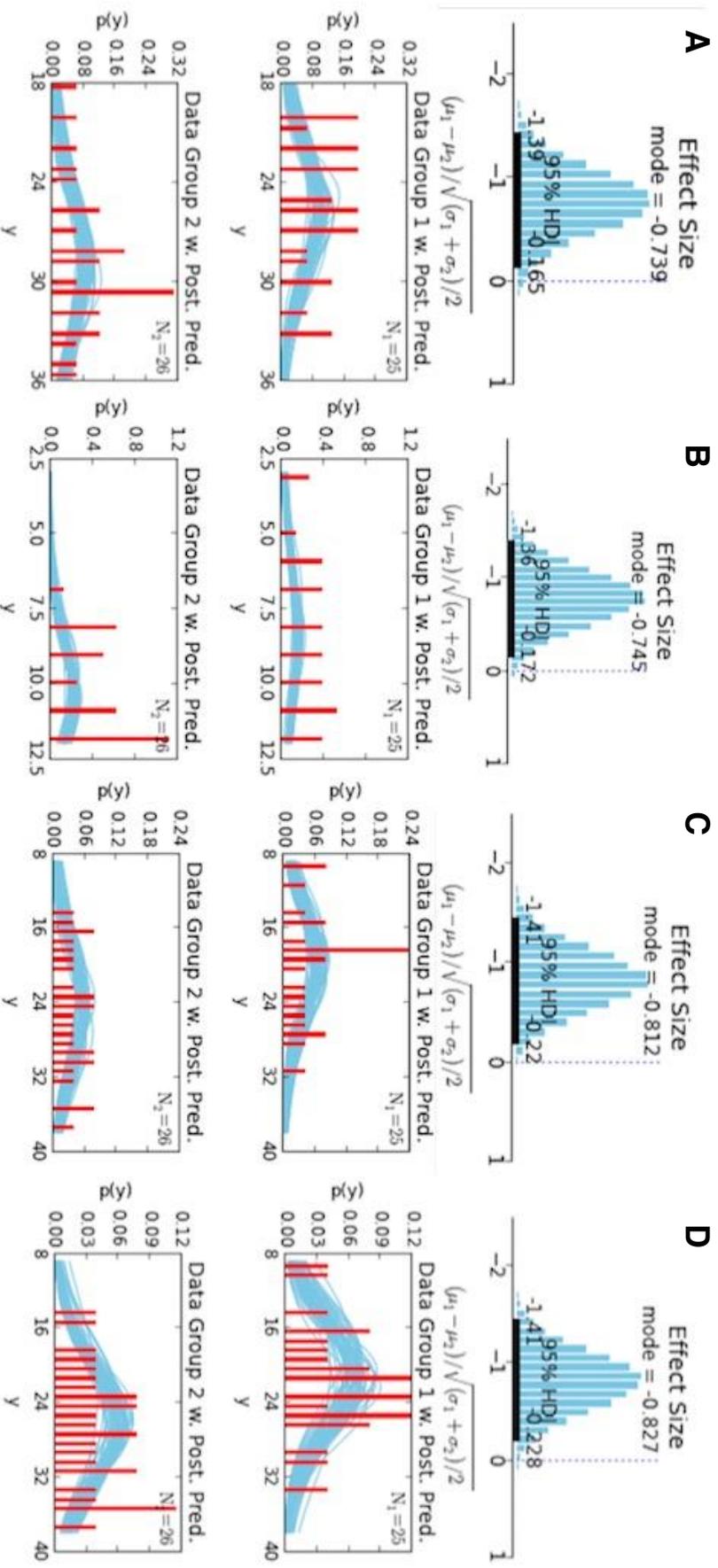
**Figure A(ii)**



Note. Figure A(ii) Indicates the BEST with MCMC simulations of significant differences in the verbal memory tests for the SAD with EDT group compared with controls indicating: A *Hopkins verbal learning immediate recall test (BEST Effect Size -0.739, 95% HDI [-0.165, -1.39]);* B *Hopkins verbal learning delayed recall test (BEST Effect Size -0.745, 95% HDI [-0.172, -1.36]);* C *Weschler memory scales logical memory delayed recall (BEST Effect Size -0.812, 95% HDI [-0.22, -1.41]); and* D *Weschler memory scales logical memory immediate recall (BEST Effect Size -0.827, 95% HDI [-0.228, -1.41]).*

238
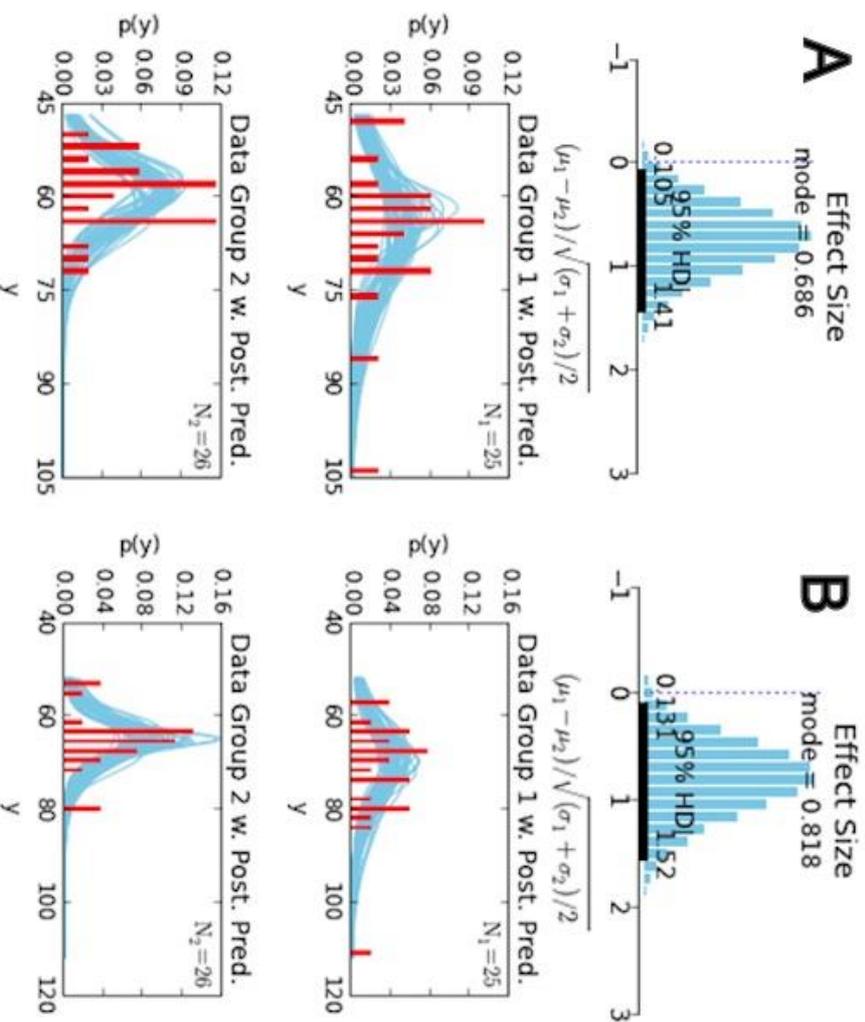
**Figure A(iii)**



Figure A(iii) Indicates the BEST with MCMC simulations of significant differences in the fine motor tests in the SAD with EDT group compared with controls indicating: A *Grooved pegboard dominant hand (BEST Effect Size -0.686, 95% HDI [1.41, 0.105]);* B *Grooved pegboard non-dominant hand (BEST Effect Size -0.818, 95% HDI [1.52, 0.131]).*
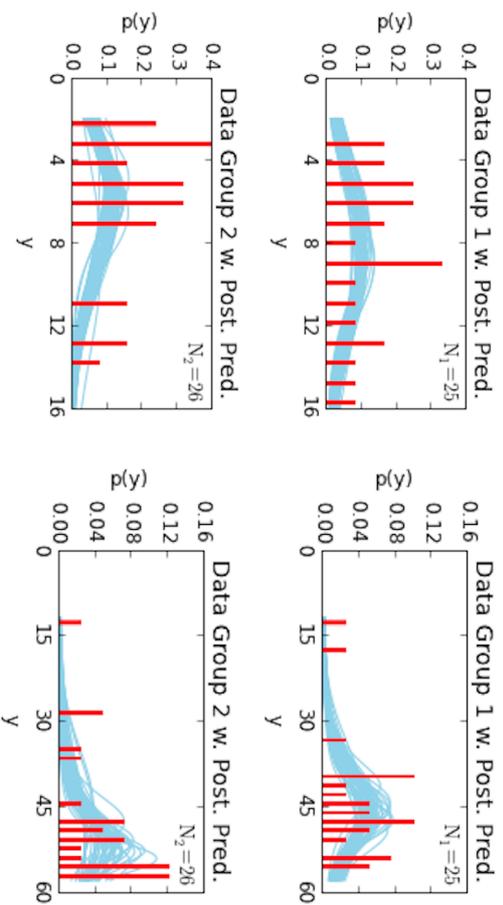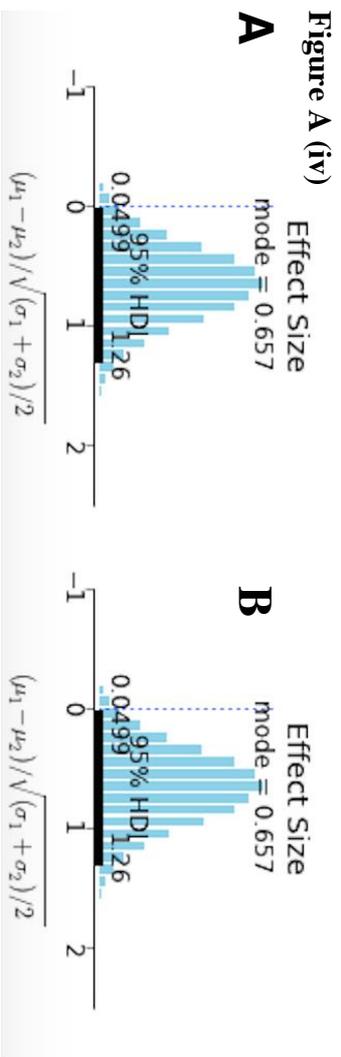
**Figure A (iv)**



Figure A(iv) Indicates the BEST with MCMC simulations of significant differences in the executive functioning tests in the SAD with EDT group compared with controls, indicating: A *Stroop test Color only* (*BEST Effect Size -0.716, 95% HDI [-0.0639, -1.28]*); **B** *Stroop test Words and Color* (*BEST Effect Size -0.752, 95% HDI [-1.35, -0.148]*); **C** *Wisconsin Card Sorting Test Conceptual Level Response* (*BEST Effect Size -0.79, 95% HDI [-1.7, -0.018]*); and **B** *Wisconsin Card Sorting Test Non-perseverative errors* (*BEST Effect Size 0.657, 95% HDI [0.0499, 1.26]*).

240

# Appendix B

## Comparisons of the outcomes of neurocognitive differences between SAD groups
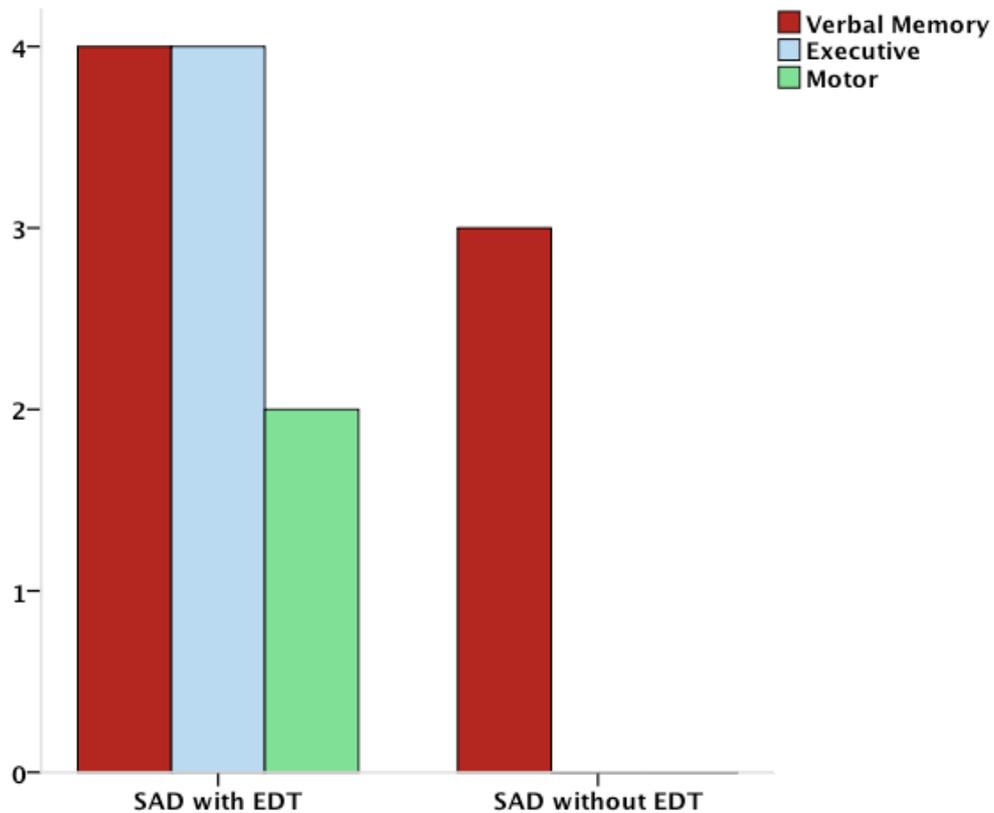


Figure B(i) shows neurocognitive differences between SAD groups: indicating the number of statistically significant neurocognitive test differences in each SAD group that was different from healthy controls. The Y-axis of the figure indicates the number of neurocognitive tests the group performed more poorly in. The SAD without EDT group only displays impairments in memory. There are a greater number of memory impairments in the SAD with EDT group compared with controls. The SAD with EDT group also demonstrates impairments in motor functioning and executive functioning compared with controls.

# Appendix C

**Location of the voxel in 3 axes for $^1$H-MRS study and data**
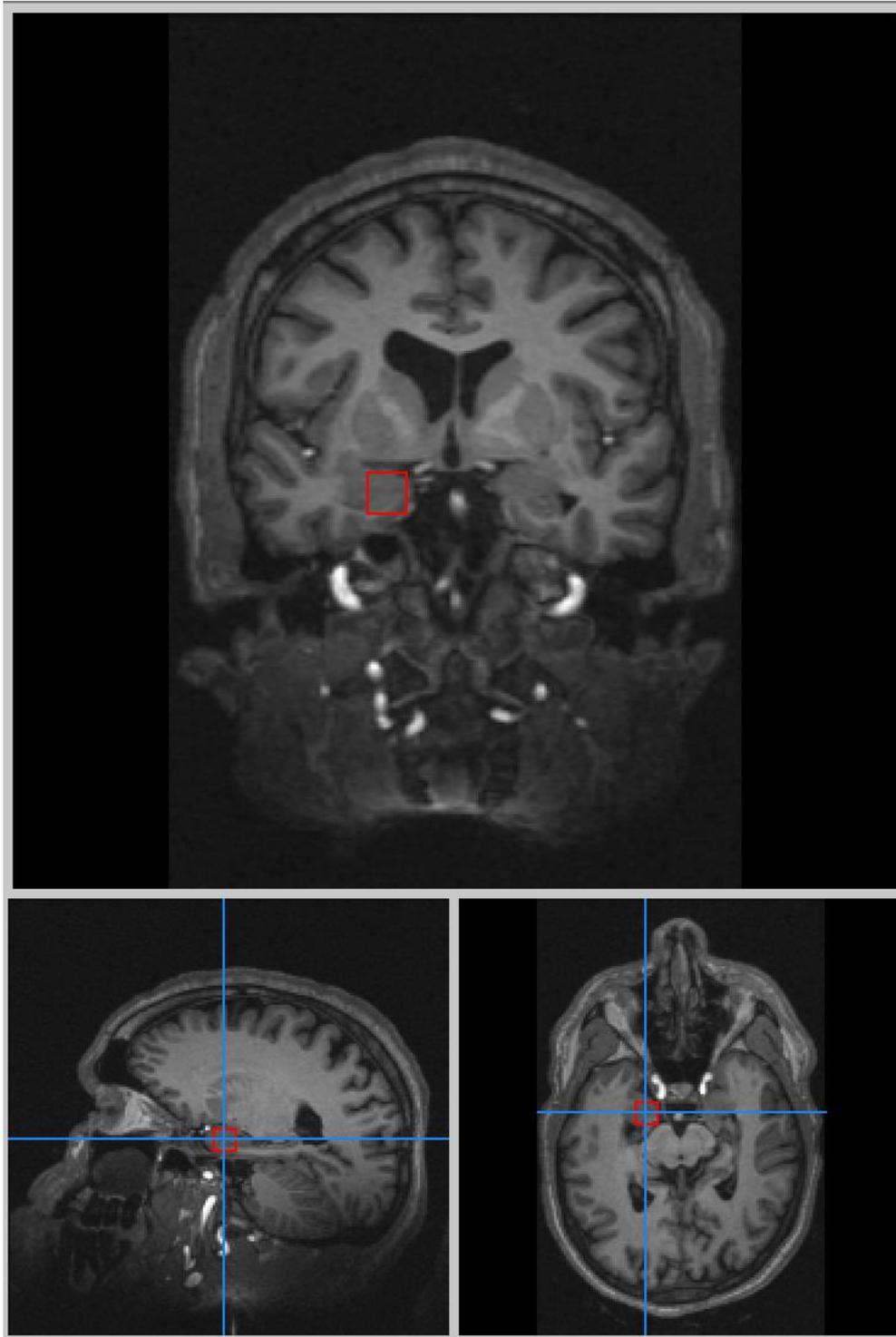


**Figure C(i)** Left amygdala single voxel $^1$H-MRS voxel placement, in three planes: saggital, coronal and axial (10 x 12 x 12

**Bayesian analyses of 1H-MRS data**

**Appendix D**

**A**

Effect Size
mode = 0.773

$(\mu_1 - \mu_2)/\sqrt{(\sigma_1 + \sigma_2)/2}$

0.112   95% HDI   1.52

**B**

Effect Size
mode = 0.768

$(\mu_1 - \mu_2)/\sqrt{(\sigma_1 + \sigma_2)/2}$

0.0861   95% HDI   1.48

**C**

Effect Size
mode = 0.725

$(\mu_1 - \mu_2)/\sqrt{(\sigma_1 + \sigma_2)/2}$

0.0256   95% HDI   1.53

**D**

Effect Size
mode = 1.21

$(\mu_1 - \mu_2)/\sqrt{(\sigma_1 + \sigma_2)/2}$

0.496   95% HDI   196

**E**

Effect Size
mode = -0.813

$(\mu_1 - \mu_2)/\sqrt{(\sigma_1 + \sigma_2)/2}$

-1.52   95% HDI   -0.165

**F**

Effect Size
mode = -0.808

$(\mu_1 - \mu_2)/\sqrt{(\sigma_1 + \sigma_2)/2}$

-1.51   95% HDI   -0.135
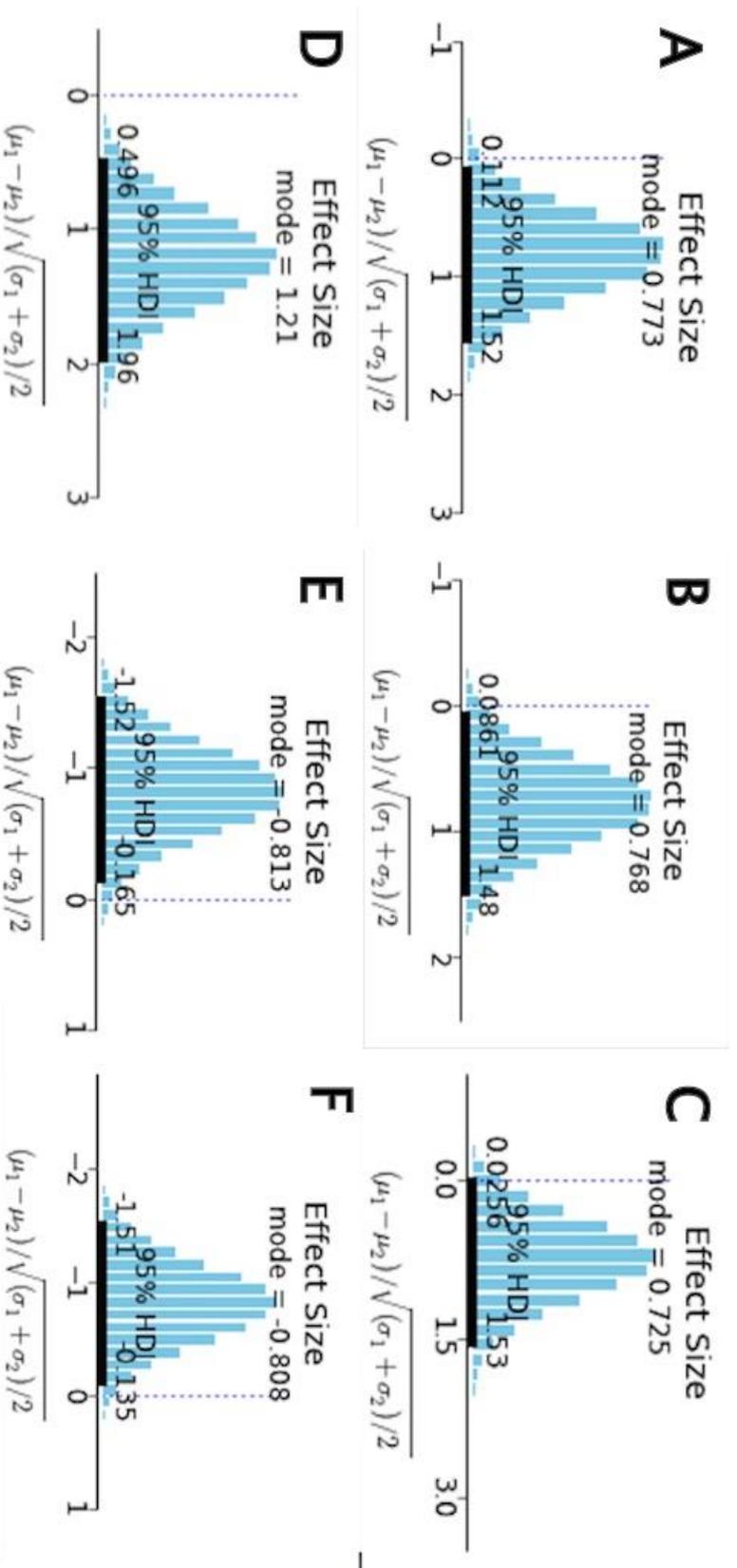
Figure D(i) represents neurometabolite BEST findings with group differences for each neurometabolite: **A** is the BEST output for Gln indicating a significant difference with greater Gln in the SAD without EDT group compared with controls; **B** and **C** is the BEST output for Glx indicating significant differences with raised Glx in the SAD with EDT and SAD without EDT groups compared with controls; **D** is the BEST output for Ins indicating a significant difference with greater Ins in the SAD with EDT group compared with controls; **E** is the BEST output for NAA indicating a significant difference with lower NAA in the SAD without EDT group compared with controls; **F** is the BEST output for **PCr** which was excluded in the final analysis and reporting for theoretical reasons for including PCr.
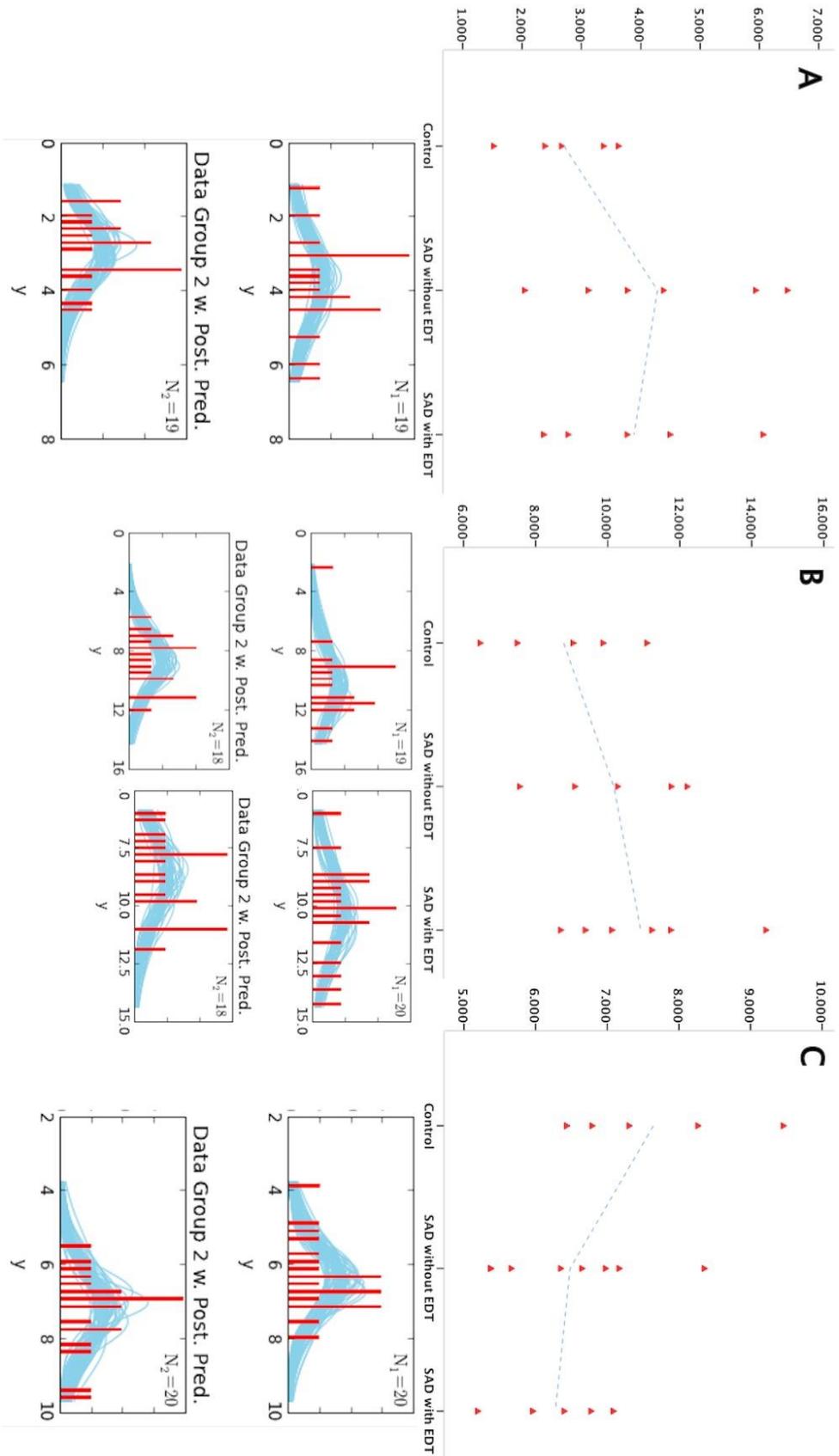
Figure D(ii) represents graphs of neurometabolites with MCMC: The graphs demonstrate the differences in means with standard deviations between each group and the respective neurometabolite, the MCMC is a reference to each BEST run for each neurometabolite, for example **A** is the graph differences and MCMC for Gln, **B** is for Glx, and **C** is for NAA for each group.

244

**D**

Data Group 2 w. Post. Pred.
$N_2 = 19$

$N_1 = 20$

**E**

Data Group 2 w. Post. Pred.
$N_2 = 20$

$N_1 = 20$
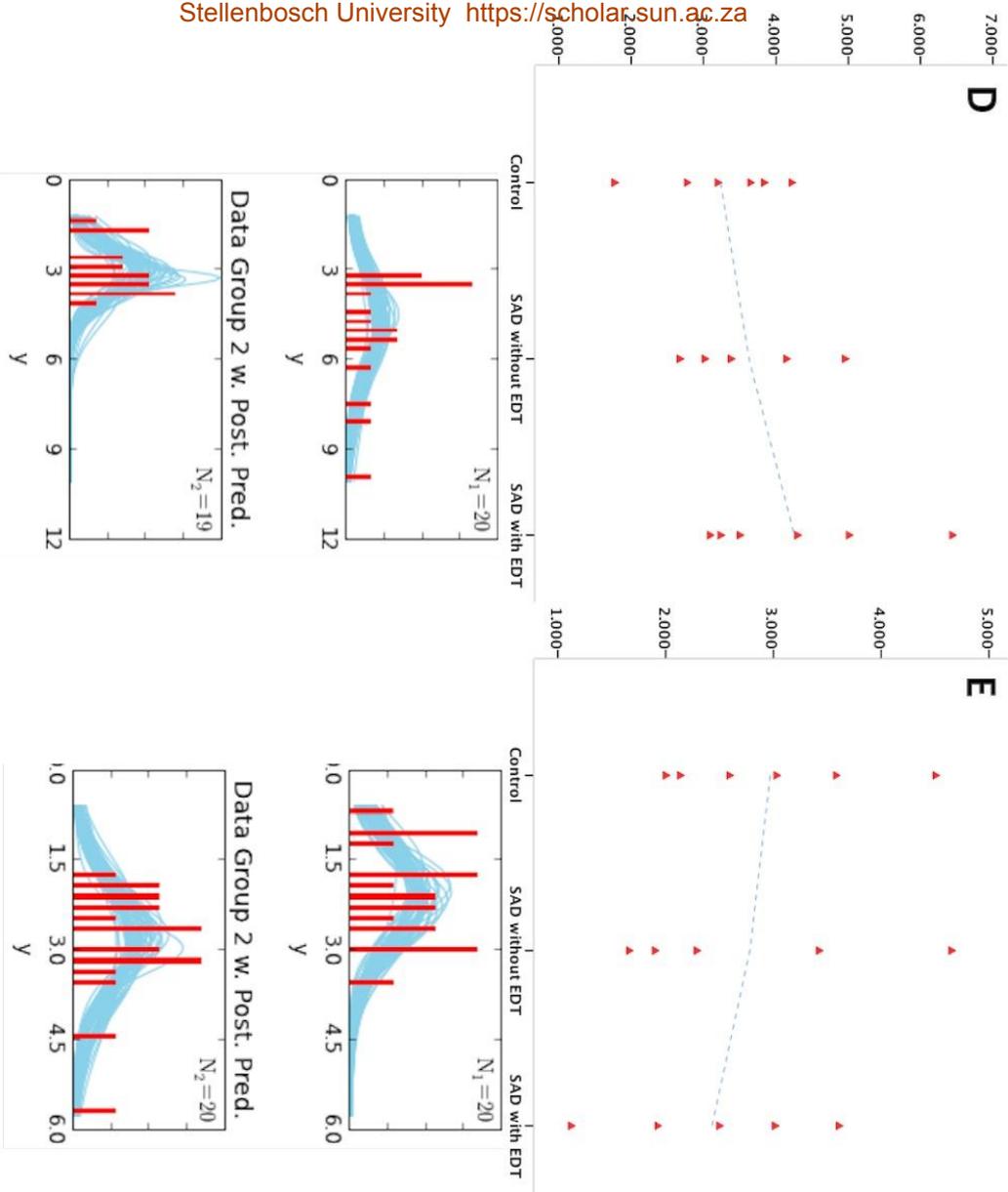
*Continuation of graphs of neurometabolites with MCMC:*

Figure D(ii cont.) shows graphs of the differences in means with standard deviations between each group and the respective neurometabolite and the MCMC's for **D**, which is for Ins and **E** is for PCr for each group.

245

# Appendix E

**Summary of the similarities and differences between the SAD groups in comorbidity, grey matter volumes and neurometabolites**

| | SAD without EDT | SAD with EDT |
|---|---|---|
| Comorbidity | MDD<br>GAD | MDD<br>GAD |
| Grey Matter Volume Differences | None | Left Thalamus [BEST (Effect Size -0.651, 95% HDI [-0.0154, -1.25]); Left ACC [BEST (Effect Size -0.758, 95% HDI [-0.203, -1.33])] Right ACC [BEST (Effect Size 0.589, 95% HDI [1.21, 0.0427])] |
| Neurometabolite Differences | Glu (Bayes Factor t = -2.530, JZS BF = 0.3068417); BEST (Effect Size 0.579, 95% HDI [0.112, 1.52)]<br><br>Glx [BEST (Effect Size 0.725, 95% HDI [0.0256, 1.53)] | Ins (Bayes Factor t = -4.084, JZS BF = 0.008294412); BEST (effect Size 1.21 95% HDI [0.496, 1.96]). NAA (Bayes Factor (t = 2.862 JZS BF = 0.1555764);<br><br>Glx [BEST (Effect Size 0.768, 95% HDI [1.48, 0.0861])] |

*Note*. Each column represents principle data findings that were statistically significant according to Bayesian analysis. These findings represent the neuroimaging and comorbidity differences between both the SAD groups and the healthy controls. The findings do not represent direct statistical comparisons between the SAD groups themselves.

# Appendix F

## Power analysis using G*Power 3

F tests - Linear multiple regression: Fixed model, R² deviation from zero
Number of predictors = 5, Effect size f² = 0,15, α err prob = 0,05