GENETIC AETIOLOGY OF ANXIETY DISORDERS

by
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Declaration

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Abstract

Anxiety disorders are among the most prevalent psychiatric disorders among both adults and adolescents. Comorbidity with other psychiatric disorders, including other anxiety disorders, is common and it is clear that a high degree of burden of distress and impairment is associated with the condition. Substantial evidence has been presented to suggest a strong genetic component in the aetiology of anxiety disorders. Twin and family studies suggest that panic disorder, general anxiety disorder, phobias and obsessive-compulsive disorder (OCD) aggregate in families. Twin studies in particular shown greater intrapair resemblance between monozygotic twins compared to dizygotic twins, suggesting a strong genetic component. Several genes have been implicated in the genetic aetiology of anxiety disorders, the most prominent of which are *BDNF* and *SCL6A4*. Furthermore, the role of the HPA axis in the regulation of the normal response to fear and stress may be influenced by genes contributing to cortisol functions such as *FKBP5* and *CRHR1*. The severity of childhood trauma can contribute to the development of anxiety disorders by modulating gene expression. In this study anxiety sensitivity (AS) is investigated as a possible predictive marker for development of anxiety disorders. Adolescents (13-18 years of age) were recruited from senior secondary schools in the Cape Town area of the Western Cape. Participants were subjected to psychological screening, which included the childhood anxiety sensitivity index (CASI) as well as the childhood trauma questionnaire (CTQ), and saliva samples were collected and genotyping conducted. Gene-environment (G × E) interactions, focussing on the severity of childhood trauma and selected genetic variants, were investigated to determine how levels of AS in a South African adolescent population were modulated. Our cohort consisted of predominantly Xhosa and Coloured individuals and analysis was done on both ethnic groups separately. Significant findings in *FKBP5* and *CRHR1* in males of both ethnic groups suggests sex linked effect in genes regulating cortisol function. The severity of childhood trauma was found to modulate selected variants which is in line with previous literature. AS may be seen as a precursor to the development of anxiety- and anxiety-related disorders, and a potential clinical marker for early diagnoses of anxiety disorders.

Opsomming

Angsversteurings is een van die mees algemene psigiatriese versteurings onder beide volwassenes en tieners. Medemorbiditeit met ander psigiatriese versteurings asook medemorbiditeit onder angsversteurings is algemeen. Verder is dit duidelik dat 'n hoë graad van las van nood en gebrek verband hou met die lyding van angsversteurings. ‘n Aansienlike hoeveelheid bewyse is beskikbaar in die literatuur dat daar 'n sterk genetiese komponent as deel van die etiologie van angsversteurings bestaan. Tweeling en familie studies dui daarop dat paniekversteuring, algemene angsversteuring, fobies en obsessiewe kompulsiewe versteuring in families meer algemeen vertoon. Tweeling studies veral wys groter intra-paar ooreenkoms tussen monosigotiese tweelinge in vergelyking met disigotiese tweelinge, wat dui dat die ooreenkoms geneties is eerder as die omgewing waarin die tweelinge hul self bevind. Verskeie gene word geïmpliseer by die genetiese etiologie van angsversteurings waarvan die mees prominente gene *BDNF* en *SCL6A4* is. Verder, die rol van die HPA-as in die regulering van die normale reaksie op vrees en stres, kan beïnvloed word deur gene wat bydra tot kortisol funksie beheer soos *FKBP5* en *CRHR1*. Kinderjare trauma kan ook bydra tot die ontwikkeling van angsversteurings, asook 'n modulerende uitwerking hê op gene. In hierdie studie word angs sensitiwiteit (AS) ondersoek as 'n moontlike voorspellende merker vir die ontwikkeling van angsversteurings. Adolessente (13-18 jaar oud) is gewerf uit senior sekondêre skole in die Kaapstad-omgewing van die Wes-Kaap om aan die studie deel te neem. Deelnemers is blootgestel aan sielkundige vraelyste soos die kinderjare angs sensitiwiteit indeks (CASI) asook die kinderjare trauma vraelys (CTQ), en speeksel monsters is ingesamel en genotipering is gedoen. Geen-omgewing (G × E) interaksies, met die fokus op die erns van kinderjare trauma en gekose genetiese variante is ondersoek, om ten einde vas te stel hoe vlakke van AS in 'n Suid-Afrikaanse adolessente bevolking is gemoduleer word. Ons studie groep bestaan uit oorwegend Xhosa en Bruin deelnemers en ontleding is gedoen op beide etniese groepe afsonderlik. Beduidende bevindinge in *FKBP5* en *CRHR1* by mans van beide etniese groepe dui op 'n geslagsgekoppelde effek in gene wat kortisol funksie reguleer. Kinderjare trauma is ook gevind om sekere variante te beïnvloed wat in lyn is met die vorige literatuur bevindings. AS kan gesien word as 'n voorloper tot die ontwikkeling van angs- en-angs verwante versteurings, en dus as 'n potensiële kliniese merker gebruik kan word tot die vroeë diagnoseering van angs versteurings.

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Chapter 1

Literature Review

I. *Introduction*

I.1 Anxiety disorders

Anxiety disorders are among the most prevalent psychiatric disorders globally (Kessler et al., 2010; Lester and Eley, 2013; Wittchen et al., 2011a). Indeed, one quarter of the European population is classified as having one or more anxiety disorder (Wittchen et al., 2011), and it has been estimated that a quarter of the population in Western countries meets the criteria for an anxiety or mood disorder in a given year. In South Africa, the lifetime prevalence of mental disorders is estimated at around 30%, with the most prevalent of these disorders being anxiety disorders, estimated at around 15% (Herman et al., 2009). From the projected lifetime risk estimates, it is estimated that almost half (47.5%) of the South African population will develop a mental disorder in their lifetime (Kessler et al., 2007). Despite the prevalence of these disorders, as well as the cost and high burden of disease, only about a quarter of individuals suffering from anxiety disorders in South Africa receive any form of treatment (Seedat et al., 2008). This seems to mirror data from Europe regarding depression and its treatment, as well as data from the United States of America. According to the Depression Research In European Society (DEPRES) survey only 25% of patients with major depressive disorder receive antidepressant medication (Tylee et al., 1999), whilst the Ontario Health Survey reported underuse of services for patients with mental disorders in both the US and Canada. Women have a higher prevalence of mental disorders and approximately a 10% higher incidence than men (Wittchen et al., 2011b; Wittchen and Jacobi, 2005). Men have been reported to show higher levels of free cortisol in response to acute stress, however some studies have shown equal responses in cortisol with females experiencing more negative effects (Foley and Kirschbaum, 2010). Women report higher levels of fear, irritability, confusion and less happiness after completion of the Trier Social Stress Test (Kelly et al., 2008).

Anxiety disorders are also the most prevalent psychiatric disorders reported in adolescents and have been shown to be linked with maladaptive outcomes in later life (Legrand et al., 1999a). Many anxiety disorders can develop in childhood and may persist into adulthood if not treated. A recent meta-analysis, using data from 27 countries including South Africa, reported a pooled estimate of 13.4% of children and adolescents affected by mental disorders

of which anxiety disorders were the most prevalent, affecting 117 million children and adolescents (Polanczyk et al., 2015).

It is clear that these disorders carry a high degree of burden of distress and impairment comparable to other chronic somatic disorders (Hettema, Neale et al. 2001). However, the exact impact of these disorders at a population level is still very poorly described. Recent studies indicate that the burden of disease is higher than previously estimated and that mental health may be one of the biggest global health challenges of our time (Wittchen et al., 2011b). A paper written by Olesen et al. (2010) estimated that the cost of mental disorders in Europe was €798 billion, of which anxiety disorders cost € 74.4 billion.

Research into the aetiology of anxiety disorders indicates that they are multifactorial in origin, and are highly comorbid with each other (Chavira et al., 2009; Davis L et al., 2010; Gureje, 2008; King-Kallimanis B et al., 2009). Some researchers have already pointed out the fallacy of extrapolating clinical data obtained in developed countries to the South African population (Wright et al., 2011). The ethnic and cultural diversity that our country is known for presents specific challenges for predicting disease progression and how patients with anxiety disorders respond to treatment. These outcomes cannot be determined by clinical characteristics alone. Studying the aetiology of the disorder has been suggested to be the most favoured approach (Lester and Eley, 2013), as genetic and environmental factors contribute to differences in course and treatment response across patient demographics (Lester and Eley, 2013).

Data from family and twin studies implicate genetics as a contributor to the aetiology of anxiety disorders (reviewed in Smoller, 2016; Plomin et al., 2016). Indeed, several studies have reported that anxiety disorders tend to aggregate in families (Lau and Eley, 2010; Legrand et al., 1999b). This phenomenon may be accounted for by a genetic predisposition to anxiety disorders; however, the impact of environmental factors that could mediate the susceptibility to anxiety disorders cannot be discounted. Twin studies are often the model of choice used in cases where genetic and environmental influences may be skewed, and these studies facilitate the delineation of genetic and familial contributory factors. Studies such as these intend to give a heritability estimate of the particular disorder, which translates into the likelihood of developing a certain disorder based on the genetic propensity of an individual (Legrand et al., 1999a). Although there is a complex interplay between environmental factors

and genetics, in some anxiety disorders the estimated genetic contribution can approach 40% (Domschke and Deckert, 2012; Erhardt and Spoormaker, 2013). The heritability of anxiety disorders, however, is not Mendelian in nature (Craddock and Sklar, 2013). Alleles that are thought to be linked to susceptibility (for any disorder) may have a range of effect sizes, as well as different frequencies within populations, ranging from rare to common (Craddock and Sklar, 2013; Wang et al., 2005). In the South African context, these factors make undertaking genetic studies more difficult as the population is very diverse, with different population groups that do not necessarily share a common genetic ancestry.

Below is a summary table of the known anxiety disorders and a brief description of their clinical presentation according to the DSM-5 diagnostic criteria (American Psychiatric Association, 2013).

Table 1.1 DSM-5 classifications of anxiety disorders

Separation anxiety disorder	Inappropriate and excessive fear or anxiety related to separation from people to whom the individual feels attached. The anxiety exceeds what is normally expected for the given age of the individual
Selective Mutism	Children who do not initiate speech nor reciprocate any verbal communication when encountering other individuals during social interactions. Normally those with selective mutism speak only in their home with immediate family members
Specific Phobia	Excessive fear or anxiety in the presence of a particular situation or object. The fear or anxiety is also experienced nearly every time the stimulus is present
Social anxiety disorder (social phobia)	Fear or anxiety of social interactions in which the individual feels he/she may be evaluated by others

Panic Disorder	Individuals suffer recurring and unexpected panic attacks
Agoraphobia	Intense fear or anxiety experienced about two or more of the following situations: using public transportation, being in open spaces, being in enclosed spaces, standing in line or being in a crown, and being outside of the home alone
Generalized anxiety disorder	Excessive anxiety and apprehension about a number of situations or activities, which the duration and intensity of the anxiety disproportionate to the likelihood of experiencing the negative event or the impact that the event may have
Substance/Medication-induced anxiety disorder	Symptoms of panic and/or anxiety that are due to the effects of a substance such as drug abuse, medication, or toxin exposure. The symptoms develop during or soon after exposure to the substance or withdrawal from use

I.2 The HPA axis: its role in anxiety

Fear is a natural emotional response to an immediate perceived threat, whilst anxiety is experienced in anticipation of future threats (Shin and Liberzon, 2010). Both fear and anxiety are necessary for physiological preparation to threatening situations such as the fight-or-flight response or increasing muscle tension and vigilance in anticipation of a threat. Fear is a biologically adaptive physiological and behavioural response to stimuli and may represent an actual or an anticipated threat to the individual's well-being. Under conditions of normal brain function, stimuli that may represent danger or potential danger elicit a response to these threats, but also receive preferential processing by the brain (Williams et al., 2010). Anxiety is triggered by generalised and less explicit signals and involves more uncertainty as to the

expectancy of threat (Bishop 2007). It has been shown in literature that anxious individuals are more likely to interpret emotionally ambiguous cues as threatening, and will display attentional bias to signals of danger (Li et al., 2005).

The normal response to stress can be viewed as two different time responses, a quick response mediated by the autonomic nervous system (ANS), and a delayed response mediated by the hypothalamic–pituitary–adrenal (HPA) axis (Lucassen et al., 2013). The first response is the "fight-or-flight" response which readies the body for immediate action by ANS stimulation of release of epinephrine and norepinephrine. Epinephrine and norepinephrine work to increase the basal metabolic rate, increase blood pressure and respiration, and increase blood flow to the heart and skeletal muscles all in preparation for response to threatening stimuli (Lucassen et al., 2013). The hypothalamic–pituitary–adrenal (HPA) axis plays a critical role in the regulation of the long-term response to fear and stress (Lucassen et al., 2013; McVicar et al., 2014; Schatzberg et al., 2014), and as such, plays a role in pathogenesis of anxiety disorders. Figure 1 is a graphical representation of the HPA axis showing the negative feedback regulation of the axis, from adrenocorticotrophic hormone-releasing factor (CRF) and vasopressin (AVP) release from the hypothalamus onto adrenocorticotrophic hormone (ACTH) secretion from the pituitary which stimulates the release of glucocorticoids (cortisol) from the adrenal cortex. Cortisol again regulates the secretion of CRF and AVP in a dose dependent manner. Cortisol binds to mineralocorticoid (MR) and glucocorticoid (GR) receptors in the hippocampus which inhibits the secretion of corticotropin-releasing hormone (CRH). Furthermore, cortisol may also bind to GR in the anterior pituitary and inhibit the secretion of ACTH and consequently inhibit its own secretion through a negative feedback mechanism (Lucassen et al., 2013; McVicar et al., 2014; Shin and Liberzon, 2010).

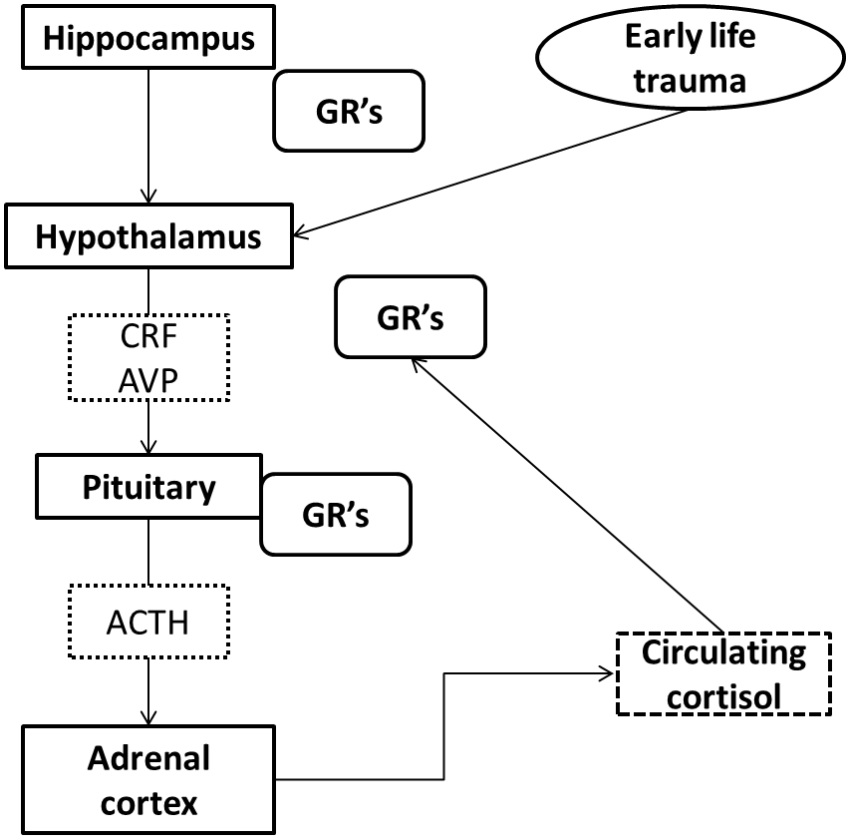

Figure 1. Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis adapted from (Pariante CM and Lightman SL, 2008). Cortisol release from the adrenal cortex is mediated through the secretion of many precursor hormones and is inhibited by its binding to receptors upstream of it secretion via negative feedback. It is hypothesized that early life trauma can affect the normal function of the HPA axis thereby contributing to the development of anxiety disorders. Abbreviations: Adrenocorticotrophic hormone-releasing factor (CRF), arginine vasopressin (AVP), adrenocorticotrophic hormone (ACTH), glucocorticoid receptors (GR).

Anxiety disorders are characterised by a persistent stimulation of the HPA axis, which can result in HPA axis dysregulation. In rodent models, adult rodents who were exposed to high levels of corticosteroids in infancy experienced hyperactivity of the HPA axis and showed altered affective behaviour similar to anxiety (Seckl JR and Holmes MC, 2007). Hyper-activation of the HPA axis can be caused by impaired signalling of GR which negates the negative feedback mechanism, leading to over expression of CRH, AVP and ACTH and eventually resulting in to increased basal cortisol levels (Ising et al., 2008). Cortisol levels in humans with PTSD have been reported to be lower than the expected norm, suggesting increased tissue sensitivity to glucocorticoids and subsequently enhance feedback mechanisms (Yehuda et al., 2004). Two genes that are described later, FK506 binding protein

5 (*FKBP5*) and Corticotropin-releasing hormone receptor 1 (*CRHR1*), have been linked to cortisol dysregulation and depression, anxiety and PTSD (Mahon et al., 2013a).

I.3 *Childhood trauma*

Childhood trauma is any action or event that causes significant harm to a child's body or psyche (DSM 5, American Psychiatric Association, 2013). This can include verbal, physical, emotional or sexual abuse, witnessing a violent act or crime, the death of a friend or relative, and any other experience that may cause trauma (DSM 5, American Psychiatric Association, 2013). Childhood trauma is a risk factor for several mental disorders and has been found to have a modulating effect on a number of genes, including sodium-dependent serotonin transporter and solute carrier family 6 member 4 (*SLC6A4*), brain derived neurotropic factor (*BDNF*), *FKBP5*, and *CRHR1* genes (Carola and Gross, 2010a; Elzinga et al., 2011; Klauke et al., 2014, 2011a; Klengel et al., 2013a; Lardinois et al., 2011; Xie et al., 2010).

Childhood trauma can be assessed using the Childhood Trauma Questionnaire (CTQ)(Bernstein et al., 1994), which is one of the most widely used measures in the trauma field. The CTQ is a 28 item self-report inventory measuring the severity of the following subscales: emotional abuse, physical abuse, sexual abuse, emotional neglect, and physical neglect. The total score provides an indication of the severity of trauma. The CTQ also assesses tendency to under report maltreatment (Bernstein et al., 1994; Villano et al., 2004). Trauma, as an environmental factor, is thought to influence genes by modification of their expression, which in turn increases the risk for development of psychiatric disorders (Klauke et al., 2011a; Lardinois et al., 2011). Genes, such as *BDNF*, *SLC6A4*, neuropeptide Y receptor 1 (*NPYR1*) and Catechol-O-methyltransferase (*COMT*), have all been shown to be influenced by childhood trauma (Baumann et al., 2013; Carola and Gross, 2010b; Klauke et al., 2011b; Wu et al., 2011). As childhood trauma has been consistently linked to the later development of psychiatric disorders, it is important to evaluate childhood trauma exposure in aetiological studies of anxiety (Table 1.2).

Table 1.2 Summary of studies looking at genetic variants and early life events with regards to anxiety disorders (Adapted from Nugent et al., 2011).

Author	**Sample Gender (M, F); Ethnicity**	**Age of ELS**	**Age at outcome M (SD) or range**	**Type of ELS**	**ELS assessment**	**Outcome**	**Outcome assessment**	**Major findings**
Blaya et al., 2010	Patients: 25,82 Controls: 37,88	Childhood	39.94 (10.17)		Childhood Trauma Questionnaire	Panic Disorder	PD diagnosis	No association between *5HTTLPR/5-HTTrs25531* and CTQ with PD
Klauke et al., 2011	100,263 EA	Childhood and adolescence	25.7 (6.7)		Childhood Trauma Questionnaire	Anxiety sensitivity	Anxiety Sensitivity Index	Carriers of 5-HTTLPR L/L genotype or 5-*HTTLPR/5-HTT rs25531* L_AL_A haplotype, in combination with high CTQ, reported increased AS
Laucht et al., 2009	142, 167 EA	Adolescence	19		Munich Events List	Anxiety and Depression	Beck Depression Inventory and Harm Avoidance subscale of the Temperament and Character Inventory	Homozygous L allele carriers of *5-HTTLPR* had higher rates of depressive or anxiety disorders.
Stein et al. (2008)	76, 171 EA	Childhood and adolescence	19 (2)	Emotional or physical abuse	Childhood Trauma Questionnaire	Anxiety sensitivity	Anxiety Sensitivity Index	Significant ELS×5-HTTLPR: greatest anxiety sensitivity (especially physical sensitivity) in s/s or s'/s' with emotional or physical abuse history
(Zavos et al., 2012b)	1556 EA	Childhood and adolescence	12-27	Dependent and independent life events	Life Event Scale for Adolescents and List of Threatening Experiences	Anxiety sensitivity	Anxiety Sensitivity Index	Anxiety sensitivity is affected by dependent and independent life events. No significant effect of *5HTTLPR* on anxiety sensitivity

Abbreviations: Childhood trauma questionnaire (CTQ), Early life stress (ELS), European Ancestry (EA), Panic Disorder (PD), The 5-hydroxytryptamine transporter-linked polymorphic region (*5HTTLPR*), 5-hydroxytryptamine transporter (5HTT).

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I.4 *Importance of adolescent studies*

Adolescence is an important developmental period where the brain is still developing and changes relating to puberty and the environment can influence susceptibility to the development of psychiatric disorders such as anxiety and depression (Comasco et al., 2015; ROMEO and M cEWEN, 2006). The mechanism behind this increased vulnerability is still unclear although exposure to stressful stimuli is thought to contribute significantly (Eiland L and Romeo RD, 2013; Turner and Lloyd, 2004). In-vivo investigation of stress and anxiety in adolescents has largely been restricted to animal models, since it is difficult to do stress related testing in young humans, as the effects of stressors at young ages are not fully known (Doremus-Fitzwater et al., 2009; Spear, 2000). Structural and functional changes have been observed in the limbic and cortical regions of the developing adolescent brain (Giedd et al., 1999; Gogtay N et al., 2004). Furthermore, changes in the reactivity of the HPA axis in response to stressors have been observed in models using adolescent rodents (Cruz et al., 2008; Romeo et al., 2006). These studies have also found a variation in corticosterone (cortisol in humans) between male and female rodents (Doremus-Fitzwater et al., 2009). It is clear that there are major differences between adolescent and adult brains, however these differences are mainly known in rodents. In this regard, it is imperative for the identification and early intervention of anxiety in youth that human studies be undertaken (Eiland L and Romeo RD, 2013; Giedd JN, 2008; Wiggins et al.,2014)

I.5 *Endophenotypes of anxiety disorders*

Endophenotypes are described as biologically informed, quantifiable intermediate phenotypes that are more closely related to the genotype than the more complex phenotypes of the disorder (Gottesman and Gould, 2003). Endophenotypes must segregate with the illness in the general population, they must be heritable, they must manifest whether the illness is present or not, they should co-segregate within families, and should present at a higher rate in families with the illness. Finally an endophenotype should be measured reliably and be specific to the illness in question (Beauchaine, 2009; Gottesman and Gould, 2003).

Psychiatric disorders are highly comorbid and comorbid disorders may be difficult to tease apart. This is especially true for anxiety disorders (Gureje, 2008; King-Kallimanis B et al., 2009) It thus follows that the search for genetically informed endophenotypes can be

immensely useful in unmasking the aetiology of complex disorders, such as anxiety disorders. A substantial body of literature exists to describe the relationship of certain anxiety-related traits, such as anxiety sensitivity (AS) and trait anxiety (TA) (Grant et al., 2007; McNally, 1989; Naragon-Gainey, 2010a; Olatunji and Wolitzky-Taylor, 2009; Plehn and Peterson, 2002; Schmidt et al., 1997; Taylor, 1995; Zavos et al., 2012c) with psychopathology. This is especially true for anxiety sensitivity. No study to our knowledge has confirmed AS or TA as endophenotypes, however they may be considered candidates based on the criterion described by Gottesman and Gould (2003). These traits are heritable (Legrand et al., 1999b), they are measurable in groups without anxiety disorders (Lambert et al., 2004), they are found in family members (Drake and Kearney, 2008), and they can be measured reliably (McNally, 1989).

I.6 *Anxiety Sensitivity*

Individuals classified as anxious live in a state in which they perpetually focus on a future likelihood of experiencing some aversive emotional or physical sensation (Paulus and Stein, 2006). Within cognitive-behavioural models, anxiety sensitivity (AS) is described as the tendency of individuals to view the experience of anxiety or other negative emotional states with fear (Paulus and Stein, 2006)(Naragon-Gainey, 2010b). Anxiety sensitivity is often referred to as an anxiety amplifier – anxious symptoms induce the fear within the individual (they become anxious due to their anxious feelings). Individuals with high AS are thought to perceive arousal of the autonomic system, such as increased heart rate, as an indicator of impending harm, and as a result, experience anxiety (they may also potentially experience panic attacks) (Schmidt et al., 2010, 2006a)

High AS has been linked to an increased likelihood of developing an anxiety disorder, such as panic disorder (Plehn and Peterson, 2002). Panic attacks are thought of as causing a conditioning of anxiety which leads to the individual fearing a recurrence of the panic attack and therefore increasing levels of AS, but also inversely relating to the fact that high levels of AS increases the likelihood of reoccurrence of a panic attack (Paulus and Stein, 2006). AS is not, however, limited to increased susceptibility to panic disorders; many disorders, such as anxiety, depression, phobias, hypochondriasis and substance abuse have all been found to have elevated levels of AS (Naragon-Gainey, 2010b)(Olatunji and Wolitzky-Taylor, 2009).

The Anxiety Sensitivy Index (ASI) is a widely used tool to measure AS (Peterson and Heilbronner, 1987). The original and standard ASI is a 16-item index that measures the concern a person has about potentially negative outcomes of having anxiety symptoms (Olatunji and Wolitzky-Taylor, 2009). AS is characterised by a hierarchical structure, with three lower-order factors, physical symptoms (fear of experiencing anxiety), publically observable anxiety symptoms (fear that others will notice the anxiety) and cognitive dyscontrol (fear of losing one's mind), loading on the higher order construct of AS : (Olatunji and Wolitzky-Taylor, 2009)(Naragon-Gainey, 2010b). Anxiety sensitivity in children and adolescents is assessed using the Childhood Anxiety Sensitivity Index (CASI) which is a formatted version of the ASI that is set up in a more child friendly manner (Silverman et al., 1991). Since its conception in 1991, the CASI has been shown to have incremental validity and has been confirmed to be as effective as the ASI, validating its use in children and adolescents (Essau et al., 2010; Lambert et al., 2004; Schmidt et al., 2010).

I.7 *Genetics of AS*

Strong evidence from twin studies indicate that familial aggregation occurs with regards to anxiety disorders; however, results have been inconsistent in many cases. The source of this familial aggregation can be strongly suggested to be genetic in origin, specifically indicated by twin studies, in which greater intra-pair resemblance between monozygotic versus dizygotic twins is due to genetic similarity rather than environmental similarity (Hettema, Neale et al. 2001). Family studies have shown a three- to five-fold increase in the risk for development of anxiety disorders if a first-degree relative suffers from panic disorder, generalized anxiety disorder or specific phobias (Domschke, 2013). One of the obvious draw backs to these types of twin studies are the size of samples included in studies (Hettema et al., 2005). Anxiety Sensitivity has been shown to be a risk factor for a number of affective disorders such as panic disorder, generalized anxiety disorder and depression (Stein et al., 2007a). AS has also been put forward as a potential intermediate phenotype for anxiety disorders (Schmidt et al., 2006b). Twin studies have reported heritability for these disorders (Eley et al., 2007) and there is evidence that AS is a core component of this phenotype (Naragon-Gainey, 2010b; Olatunji and Wolitzky-Taylor, 2009; Stein et al., 2007a; Zavos et al., 2012c). Heritability of AS is estimated to be around 50% in adults (Stein et al., 1999),

37% in childhood (Eley et al., 2007), and 47% in adolescence (Zavos et al., 2010). AS has been found to be phenotypically stable, meaning that AS measured in adolescence is often similar to AS measured in adulthood (Zavos et al., 2012a). Levels of childhood maltreatment have been shown to influence AS in the context of certain genetic variants such as *5HTTLPR* (Stein et al., 2007b).

I.8 *Genetic aetiology of anxiety disorders*

The serotonin transporter gene (*SLC6A4*) and *BDNF*, are two of the most widely studied with regards to the association of specific SNPs (single nucleotide polymorphisms) in these genes and anxiety and stress-related disorders (e.g. post-traumatic stress disorder (Martinowich et al., 2007a; Montag et al., 2010; Schmidt and Duman, 2010). The serotonin transporter, also known as *SLC6A4*, has been well documented in terms of its function in regulating serotonin levels in the brain. The *SLC6A4* gene has polymorphisms in its promoter region that affects the transcription of the serotonin transporter and its subunits (Klauke et al., 2011b; Nordquist and Oreland, 2010; Smits et al., 2008; Uher and McGuffin, 2007). The 5-hydroxytryptamine transporter-linked polymorphic region (*5HTTLPR*) gene has been implicated in the aetiology of many psychiatric disorders, such as depression, anxiety, schizophrenia, substance abuse disorders, autism spectrum disorders, and others (Nordquist and Oreland, 2010)(Plieger et al., 2014). A great body of evidence speaks to serotonin's function in determining behavioural traits as well as its role in the aetiology of several psychiatric disorders (Guiard et al., 2008; Klauke et al., 2011b; Miller et al., 2009; Nordquist and Oreland, 2010; Stein et al., 2007a; Uher and McGuffin, 2007; Zavos et al., 2012c). While there is a growing body of evidence pointing toward a functional correlation to the *5HTTLPR* genotypes (Zavos et al., 2012c), some studies have also shown no link between the serotonin transporter gene and anxiety disorders (Chorbov et al., 2007; Jorm et al., 1998a; Laucht et al., 2009b; Power et al., 2010a). Findings have been inconsistent, in part because of the complex nature of polymorphisms and alleles within this gene. The *5HTTLPR* is a variable number tandem repeat (VNTR) marker of which there are two alleles of interest to this study , a short "*S*" allele, which has been associated with decreased serotonin transporter expression (Uher, 2008) and therefore decreased so called "positive" outcomes (Caspi et al., 2003; Karg et al., 2011; Uher and

McGuffin, 2007). The long "*L*" allele has been shown to be linked to increased serotonin transporter expression and "anxiety-like traits".

Brain derived neurotrophic factor (*BDNF*) is a protein involved in the regulation of neural growth and stimulation of synaptic plasticity. BDNF belongs to a family of neurotrophic factors that influence proliferation, migration, differentiation, growth and apoptosis of mammalian neural cells (Hashimoto K, 2007). Recently *BDNF* has become a focal point for research into depression and anxiety (Montag et al., 2010). Data suggests that *BDNF* could play a modulatory role in the experience-dependent programming of anxiety. Certain polymorphisms in *BDNF*, such as the Val66Met variant, have been found to mediate altered anxiety-like behaviour in rat models; however not all results are consistent (Domschke et al., 2010; Elzinga et al., 2011; Martinowich et al., 2007a; Montag et al., 2010, 2008; Schmidt and Duman, 2010). Rats with the non-synonymous Val66Met polymorphism have been found to be more sensitive towards early life events, with stressors at early development leading to greater risks of anxiety and depression in later life stages (Carola and Gross, 2010b). These discrepancies may point to a greater environmental influence on BDNF function and its role in anxiety.

Exposure to stressors has been shown to alter expression of *BDNF* in the hippocampus (Schmidt and Duman, 2010), whilst chronic use of antidepressants increases expression of *BDNF* in these brain regions. *BDNF* expression and signalling seems to play an important role in the normal response to antidepressants as a variety of BDNF deficient mice show little or no behavioural response to antidepressant administration (Schmidt and Duman, 2010). However, *BDNF* mutant mice only show increases in their stressed behaviour when exposed to stressful environments. Under 'no stress' conditions the knockout of *BDNF* function seems to have no effect on behaviour. This suggests that *BDNF* does not cause depressive or anxious behaviour with its absence, but rather the lack of proper *BDNF* expression leads to decreased coping mechanisms in response to stressful life events (Domschke et al., 2010; Schmidt and Duman, 2010).

BDNF is a potential biomarker for MDD and/or antidepressant treatment efficacy, which could also potentially mean that the genetic variations in the *BDNF* gene could serve as markers of other affective disorders (Schmidt and Duman, 2010). *BDNF* expression levels have been found to correlate with increased depression and anxiety in several studies. More

importantly, several studies have shown increased levels of BDNF post mortem in depressed patients who were treated with antidepressants (Martinowich et al., 2007a; Montag et al., 2010). Findings such as these have led to the hypothesis that BDNF may play some role in the recovery process from depression. In contrast to this however, several animal studies have found that infusion of BDNF to areas of the brain can lead to depressive-like behaviour (Montag et al., 2010). Conflicts in findings such as these, point toward specific functioning of BDNF within specific regions of the brain.

The critical role that BDNF plays to maintain and promote normal neuronal growth and function makes it a key point of investigation for the aetiological study of disorders of the mind. Not only is it clear that abnormal functioning of BDNF leads to deficiencies in neural upkeep, which in turn may lead to psychopathology, but also that *BDNF* is susceptible to epigenetic modifications (Elzinga et al., 2011; Martinowich et al., 2007a; Middeldorp et al., 2010). Disagreement across various studies about the specific role of *BDNF* also lends credence to the possibility that the whole picture of *BDNF* and its role in the development of anxiety disorders may be moderated by a combination of genetic and epigenetic mechanisms (Domschke et al., 2010; Elzinga et al., 2011).

Corticotrophin-releasing hormone (CRH) and CRH type 1 receptor gene (*CRHR1*) are important mediators of the stress response (Wang et al., 2012). Subsequently *CRHR1* has been thought to play a role in the pathophysiology of anxiety disorders, this is based in part on studies done on transgenic mice (Heinrichs and Koob, 2004; Reul and Holsboer, 2002). Hormonal control of stress through the HPA (Hypothalamic-pituitary-adrenal) axis is important in the long term response to stress, as well as influence autonomic response to stress under chronic conditions. A CRHR1 antagonist may act as an anxiolytic as mice that over express CRH show increased anxiety like behaviour, whilst mice that are *CRHR1* knockout show reduced levels of anxiety (Reul and Holsboer, 2002; Timpl et al., 1998; Wang et al., 2012). Early life trauma or stress has also been shown to influence the expression of *CRHR1* and specifically increases the levels of CRH that is present in the hippocampus (Fenoglio et al., 2006). This modification of the normal stress response is further exacerbated by the presence of early life trauma (Faravelli et al., 2012) adding to the complex nature of the pathogenesis of anxiety disorders.

Glucocorticoid receptors (GR) are vital for a healthy response to stress. These receptors mediate the body's natural stress response through a negative feedback control. Glucocorticoids bind to GR to activate the so called 'Fight or Flight' response, readying the body for activity and terminating the response when danger has passed. The negative feedback loop of GR is integral to normal stress response. Over-activation of this system has been linked to several mood disorders. Partial glucocorticoid resistance is observed in mood disorders due to improper signalling of GR (Binder, 2009).

GR is ligand-activated and translocates from the cytosol to the nucleus through a large molecular complex. Chaperone molecules, called heatshock proteins (hsp), allow for the proper folding of peptides and proteins within this molecular machinery. FK506 binding protein 51 (FKBP5) is a co-chaperone playing a role in the proper folding and binding of GR and has been associated with the pathophysiology of several disorders (Appel et al., 2011b, p. 5; Binder, 2009; Binder et al., 2004a, p. 5; Klengel et al., 2013a, p. 5). Polymorphisms in the *FKBP5* gene have been found to be involved in GR resistance and linked to susceptibility to the development of post-traumatic stress disorder (Xie et al., 2010). Genetic variation within the *FKBP5* gene could alter the sensitivity of the stress response pathway, especially during development, possibly putting individuals at risk for development of psychiatric disorders (Xie et al., 2010). *FKBP5* has been studied in the context of childhood adversity/trauma and its role in mediating susceptibility to disorders such as depression, PTSD and anxiety (Appel et al., 2011; Binder, 2009; Binder et al., 2004; Klengel et al., 2013; Xie et al., 2010).

I.1.9 *The present study*

Clinical and demographic indicators have proven to be ineffective as predictors of treatment outcomes (Lester and Eley, 2013), which has sparked an interest in finding biomarkers that may more accurately predict treatment responses (Lester and Eley, 2013; Bieber, 2013; Trusheim et al., 2011, 2007)). It is within this context that endophenotypes may be useful to improve diagnosis as well as potentially play a role in treatment response prediction as well. Early detection of mental disorders is preferable in order to improve clinical treatment as well as treatment outcomes. AS stands out in the literature as a robust predictive endophenotype for anxiety disorders. AS is a well-studied risk factor for anxiety disorders (Stein et al., 2007b) and has been investigated as an intermediate phenotype for anxiety disorders (Schmidt et al., 2006b). For these reasons and others as described above, AS was used in this

study as a risk marker for anxiety in adolescents. *FKBP5*, *BDNF*, *CRHR1*, and *SLC6A4* are genes that have been previously implicated in the aetiopathogenesis of anxiety disorders and have been associated with AS as well as with adverse life events (Comasco et al., 2015, p. 5; Mahon et al., 2013a; Martinowich et al., 2007b; Zavos et al., 2012c). Accordingly, these genes have been selected for investigation in our study.

Study objectives

This study aims to investigate anxiety sensitivity (AS) (measured as CASI total score) as a predictive marker of susceptibility risk for anxiety disorders in an adolescent South African cohort, considering previously identified genetic risk variants. Furthermore, the addition of environmental exposure, more specifically childhood trauma (measured by CTQ total score), will be utilized to investigate gene-environment interactions in the aetiology of AS.

The study objectives are as follows:

1. Use regression analysis to determine association between AS and selected genetic variants assuming additive allelic, dominant, and recessive models of inheritance.
2. Use regression analysis to determine whether selected genetic risk variants interact with childhood trauma to mediate the development of AS.
3. Establish a strong base of evidence for anxiety related endophenotypes in a South African cohort.

Chapter 2

Materials and Methods

II. *Materials and methods*

II.1 *Ethics*

This study falls under the ethics purview of the parent study: Relationship between childhood trauma, neuropsychological deficits, neural circuitry, and anxiety proneness in high-anxiety and low-anxiety prone adolescents. The study has been approved by Health Research Ethics Committee (HREC) of the Faculty of Medicine and Health Sciences, University of Stellenbosch (N10/11/370).

II.2 *Participants*

A total of 1149 study participants (13 – 18 years) were recruited from senior secondary schools in the Cape Town area of the Western Cape. Learners provided written and informed assent and written and informed consent was obtained from parents or guardians. The inclusion criteria for participation were as follows: (1) the ability to read, write and understand English or Afrikaans at the 5th grade level (2) psychotropic drug-naïve (3); medically sound and able to undergo psychological testing and magnetic resonance imaging (MRI) scanning. Exclusion criteria comprised: (1) prior treatment for anxiety disorders (2) current or past history of mental, psychotic or childhood disorders (3) a history of alcohol or substance abuse/dependence (4) previous head trauma (5) and currently on psychotropic medication. The ethnicities of the participants were Xhosa, Coloured, White, Asian, and other; and was determined through self-report (Table 3.1).

II.3 *Psychological screening*

All participants were screened by a trained research psychologist to determine self-reported levels of childhood maltreatment/trauma, as measured by the childhood trauma questionnaire (CTQ)(Bernstein et al., 1994), and anxiety proneness. Anxiety sensitivity, measured by the Child Anxiety Sensitivity Index (CASI)(Silverman et al., 1991) and trait anxiety, measured by the trait section of the State-Trait Anxiety Inventory (STAI)(Spielberger et al., 1970) were selected as predictive markers of anxiety. Several other screening measures were also administered to participants to determine eligibility.

Measures:

(1) The Child Anxiety Sensitivity Index (CASI) (Silverman et al., 1991):

An 18-item self-report questionnaire measuring the fear of anxiety by rating the extent to which an experience of anxiety will result in a negative consequence(s).

(2) The Childhood Trauma Questionnaire (CTQ)(Bernstein et al., 1994):

A 28-item retrospective measure of the frequency and severity of abuse and neglect experienced prior to age 18. The CTQ consists of five subscales that assess emotional, physical, and sexual abuse and emotional and physical neglect, respectively. For the purposes of the proposed study, enquiry was made into maltreatment experienced prior to age 12.

(3) The Life Events Timeline:

Participants were instructed to indicate on a timeline at which age/s major life events occurred, as indicated on the CTQ.

(4) The Center for Epidemiological Studies Depression Scale for children (CES-DC)(Weissman et al., 1980):

A 20-item self-report measure of depression symptoms experienced during the past week.

(5) The Alcohol Use Disorders Identification Test (AUDIT)(Babor et al., 2001; Saunders et al., 1993):

A 10-item self-report measure used to identify hazardous and harmful patterns of alcohol consumption during the past year, by assessing recent alcohol use, alcohol dependence symptoms and alcohol-related problems.

(6) The Drug Use Disorders Identification Test (DUDIT)(Berman et al., 2004) :

A 11-item self-report measure used to identify drug use patterns and various drug-related problems.

(7) The Connor-Davidson Resilience Scale (CD-RISC)(Connor and Davidson, 2003):

A 25-item self-report measure that assesses the level of stress coping ability over the past month.

(8) Adolescent Coping Orientation for Problem Experiences (A-COPE)(Patterson and McCubbin, 1987):

A 54-item self-report coping inventory used to measure the behaviour and patterns adolescents find helpful in managing problems or difficult situations.

II.4 *Sample collection and DNA extraction*

Of the 1149 participant, 986 saliva samples were collected using Oragene collection tubes (DNAGenotek, Canada) and stored at room temperature. DNA was extracted using the PrepIT-L2P DNA extraction kit (DNAGenotek, Canada) as per manufacturer's instruction and suspended in 100 µl Tris EDTA (TE) solution for storage at -80 °C. DNA concentration was assessed using the Nanodrop (Delaware, USA). DNA concentration was assessed at an absorbance maximum of 260nm (A_{260}) and sample purity assessed using the A_{260}/A_{280} ratio. A ratio of >1.8 was used as a cut-off for inclusion in this study, indicative of low protein contamination. DNA samples were stored at -80^0C.All samples were diluted to a concentration of 80 ng/µl using TE buffer. Samples with concentrations below 80 ng/µl were not diluted, but concentrations were noted.

II.5. *Polymorphism selection*

Four candidate genes were prioritised based on recent literature and relevance to anxiety disorders. Polymorphisms within these genes were selected using a TagSNP approach as well as a literature search focusing on (1) reported functionality of the SNP and (2) relative position in the gene (i.e exon vs. intron vs. regulatory region). Polymorphisms with minor allele frequencies (MAFs) ≥ 0.2 were included in the study to select for common variation (MAF according to HapMap (NCBI build 36, dbSNP b126) (www.hapmap.org). MAF selection was based on CEU (Utah residents with Northern and Western European ancestry from the CEPH collection), MKK (Maasai in Kinyawa, Kenya), and YRI (Yoruban in Ibadan, Nigeria). If an allele was present at the minimum MAF in all of the above mentioned populations, they were included as there is no reference ancestry information available for the

South African population a combination of populations was used. A list of genes, selected polymorphisms and reported MAFs can be found in Table 2.1 below.

Table 2.1. List of selected candidate genes and polymorphisms with accompanied minor allele frequencies and chromosome locations.

Gene	rs#	Polymorphism type	Assembly	Chromosome location	MAF	Genotype Technique
FKBP5	rs3800373	SNV	**GRCh38**	6:35574699	0.325	Sequenom
FKBP5	rs9296158	SNV	**GRCh38**	6:35599305	0.359	Sequenom
FKBP5	rs737054	SNV	**GRCh38**	6:35607710	0.193	Sequenom
FKBP5	rs6926133	SNV	**GRCh38**	6:35611598	0.192	Sequenom
FKBP5	rs1360780	SNV	**GRCh38**	6:35639794	0.373	Sequenom
FKBP5	rs9394309	SNV	**GRCh38**	6:35654004	0.247	Sequenom
FKBP5	rs9470080	SNV	**GRCh38**	6:35678658	0.363	Sequenom
BDNF	rs11030099	SNV	**GRCh38**	11:27656036	0.229	Sequenom
BDNF	rs6265	SNV	**GRCh38**	11:27658369	0.201	Sequenom
BDNF	rs2049046	SNV	**GRCh38**	11:27702228	0.43	Sequenom
SLC6A4	rs3813034	SNV	**GRCh38**	17:30197786	0.483	KASP
SLC6A4	rs1042173	SNV	**GRCh38**	17:30197993	0.485	Sequenom
SLC6A4	rs6354	SNV	**GRCh38**	17:30222880	0.204	Sequenom
SLC6A4	rs25531	SNV	**GRCh38**	17:30237328	0.138	Manual genotyping
CRHR1	rs7209436	SNV	**GRCh38**	17:45792776	0.443	Sequenom
CRHR1	rs4792887	SNV	**GRCh38**	17:45799654	0.13	Sequenom
CRHR1	rs110402	SNV	**GRCh38**	17:45802681	0.439	Sequenom
CRHR1	rs242924	SNV	**GRCh38**	17:45808001	0.436	Sequenom
SLC6A4	5HTTLPR	DRPR	**GRCh38**	17q11.2	N/A	Manual genotyping

MAF were determined using dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). All MAFs were verified using multiple population groups through the HapMap database (http://hapmap.ncbi.nlm.nih.gov/index.html.en). MAF above according to CEU population. Abbreviations: Single nucleoitide variation (SNV), Degenerate repeat polymorphic region (DRPR), Sequenom's iPLEX® Gold assay (sequenom), LGC genomics KASP technology (KASP), FK506 binding protein 5 (*FKBP5*), brain derived neurotropic factor (BNDF), sodium-dependent serotonin transporter and solute carrier family 6 member 4 (*SLC6A4*), and corticotropin-releasing hormone receptor 1 (*CRHR1*).

II.6. *Polymerase Chain Reaction (PCR)*

II.6.1. *Primer design*

Primer design for the serotonin transporter gene promoter region (*5HTTLPR*) was obtained from Voyiaziakis et al. (2009) and synthesized by Synthetic DNA Laboratory (Molecular and Cell Biology, University of Cape Town). All other primers were designed, synthesized and wet bench tested by Genome Quebec (Canada) and LGC Genomics (United Kingdom) respectively.

II.6.2. *PCR conditions*

II.6.2.1. Serotonin transporter gene (*SLC6A4)*, rs25531

5HTTLPR primers were labelled at the 5' end as follows: Fluorescently labelled FAM (6-fluorescein amidite) forward primer: FAM-5' *ATG CCA GCA CCT AAC CCC TAA TGT* 3' and un-labelled reverse 5' *GGA CCG CAA GGT GGG CGG GA* 3'.

PCR conditions for in-house genotyping of *5HTTLPR* were performed according to the manufacturer's instructions. KAPA 2G Robust HotStart readymix was used for *5HTTLPR*. PCR conditions for all subsequent genotyping, outsourced to Genome Quebec and LGC Genomics were based on standard protocols for Sequenom and KASP technologies. PCRs were performed in 25µl reaction volumes of which 9 µl was PCR grade water, 12 µl Kapa2G Robust HotStart ReadyMix, 1.25 µl forward primer, 1.25 µl reverse primer, and 1 µl sample DNA. PCR conditions were as follows: initial denaturation at 95°C for 3 minutes, followed by denaturation at 95°C for 15 seconds cycling 35 times, primer annealing at 60°C for 15 seconds for 35 cycles, extension at 72°C for 15 seconds for 35 cycles, final extension at 72°C for 10 minutes, and finally cooling at 4°C until removed.

II.7. *Genotyping*

The genotyping methods used were as follows (Table 2.1):

1. Microsatellite length polymorphism analyses
2. Restriction fragment length polymorphism (RFLP) analyses
3. KASP® Genotyping
4. Sequenom genotyping

Subsequent to *5HTTLPR* amplification, PCR success was assessed by electrophoresing 5 ul of each amplicon on a 2% (w/v) agarose gel. Once complete, the gel was viewed under ultraviolet light. A successful PCR yielded bands of 280 bp (short, *S*-allele) and/or 320 bp (long, *L*-allele).

The remaining product was genotyped for rs25531 using a two-stage protocol. The first stage involved allele-specific restriction enzyme analysis (ASREA), making use of the *MspI* restriction enzyme as per the manufacturer's protocol. Restriction enzyme (RE) digest was done in PCR tubes at a constant temperature of 37°C for 12 hours. Reaction volumes were as follows: 5 µl of PCR product, 1 µl Thermo Scientific Tango Buffer, 0.25 µl *MspI* enzyme, and 3.75 µl of PCR grade water totalling a reaction volume of 10 µl. This allows for the genotyping of rs25531 through the use of capillary electrophoresis which was carried out at the Central Analytical Facility (CAF) at Stellenbosch University. Prior to capillary electrophoresis 5µl of product was used for electrophoresis on a 2.5% agarose gel to ensure that the digest was complete.

The *Mspl* restriction enzyme has a 5' *CCGG* 3' recognition site, and rs25531 is an ***A***>***G*** SNP. This means that if a ***G*** allele is present, the restriction enzyme will cut the *5HTTLPR* amplicon, however if an ***A*** allele is present, no RE digestion will occur. After digestion, a fragment of the amplicon remains FAM-labelled. This remaining fragment size, combined with the L and S fragment sizes, is used to determine the eventual combined genotype (Rs25531 alleles are indicated using lowercase a and g). The FAM-labelled fragment sizes (in bold) are as follows:

Sa/Sa → **281** + 94

La/La →**325** + 94

Sg/Sg → **151** + 130 + 94

Lg/Lg → **151** + 174 + 94

Lg/Sg → **151** + 174 + 130 + 94

Lg/Sa → **151** + 281 + 174 + 94

La/Sg → **325** + 151 + 130 + 94

Further genotyping was performed using the Sequenom's iPLEX® Gold assay (Genome Quebec, Canada).

The Sequenom's iPLEX® Gold assay (henceforth referred to as Sequenom) allows for the genotyping of up to 40 markers. This method distinguishes alleles based on different masses of primer extension products. Target regions are amplified using a PCR reaction, after which unincorporated dNTPs are inactivated. This is followed by a primer extension reaction (the iPLEX reaction) which utilizes mass modified ddNTPs (Bradić et al., 2012). Nucleotides added differ according to the allele present directly downstream of the 3' end of the primer. After a clean-up step the extended primers are transferred to a chip containing a specific matrix which allows for detection using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The only limiting factor for the design of new assays for Sequenom genotyping is suitable primers in the region of interests (Bradić et al., 2012).

KASP technology (LGC genomics, United Kingdom) was utilised to genotype those polymorphisms that could not be genotyped using the Sequenom technique.

KASP genotyping assays are the proprietary genotyping technology of LGC Genomics (UK). A SNP-specific assay mix, as well as a universal KASP mastermix, is added to samples after which a thermal cycling reaction is performed. The KASP assay mix contains two allele-specific forward primers and one common reverse primer, all of which are unlabelled. The two allele-specific primers have unique tail sequences incorporated that correspond to a

universal fluorescence resonant energy transfer (FRET) cassette. During the thermal cycling step, the allele-specific primer binds to the template and elongates, incorporating the unique tail sequence to the newly formed strand. Subsequent rounds of cycling form complements of said tails sequence which allows the FRET cassette to bind to the DNA. The tail sequence stops the quenching effect on the FRET cassette which enables it to fluoresce.

II.8. *Statistical analyses*

Demographic and clinical characteristics were summarised using means and standard deviations, if approximately normally distributed, and as medians and ranges if non-normally distributed. Differences between groups (gender, ethnicity) were assessed using unadjusted linear models, transforming traits to normality where necessary. Subscales of the CTQ total were summarised by categorising the scores, and reporting number (%) within each category. Genotype counts (%) and HWE p-values were summarised separately for Xhosa and Coloured participants.

General linear modelling was used to express CASI total score (AS) as a function of a genotype, additive allelic or haplotype variable, whilst adjusting for possible confounders. The possible confounders for association testing were age, gender, depression (CESD total score), resilience (CD-RISC total score), coping mechanisms (A-COPE total score), trait anxiety (STAIT total score), alcohol use disorders (AUDIT total score) and Childhood Trauma (CTQ total score). Genotypes were coded as three categories (2 degrees of freedom test), where alleles were coded as the number of minor alleles present (0,1 or 2). Haplotypes were also coded as the number present, and were inferred using the solid spine of linkage disequilibrium (LD) method using Haploview software (version 4.2) (Barrett et al., 2005). The only model used was additive allelic, however, controlling for the same confounding factors as for the single-locus analyses.

Modelling was done separately for Coloured and Xhosa participants. Where significance was detected ($p<0.05$) dominant and recessive minor allele models of possible inheritance models were investigated, and gender effects were analysed. A best fit approach was used to determine which of the four possible models was used to estimate effects reported. All analyses were done using R (Team, 2012), and functions from R packages *genetics* (Warnes et al., 2011), *haplo.stats* (Sinnwell & Schaid, 2012) and *effects* (Fox, 2003).

Chapter 3

Results

III. *Results*

III.1 *Clinical and demographic data*

Of the 1149 original samples taken, 985 individuals were successfully genotyped and were stratified into ethnic groups, which were identified by means of self-report. Individuals self-identified as White, Asian and other were excluded from further analysis due to the fact that there was only a total of 34 individuals in these categories. This resulted in a cohort size of 951 of which 317 self-identified as Coloured (32.3%) and 634 self-identified as Xhosa (64.3%) (Table 3.1).

Table 3.1. Ethnic distribution of participants

	Count	Percent
White	21	2.1
Coloured	317	32.2
Xhosa	634	64.4
Asian	1	0.1
Other	12	1.2
Total	985	100.0

Table 3.2. Demographic and clinical data of the Xhosa and Coloured participants.

Clinical/demographic characteristic	Xhosa			Coloured		
	Female	**Male**	**All**	**Female**	**Male**	**All**
Number	375 (59%)	259 (41%)	634	188 (59%)	129 (41%)	317
Age (years), mean (SD)	16.4 (2.1)	16.4 (2.0)	16.4 (2.1)	15.8 (1.7)	15.80 (1.5)	15.8 (1.6)
CASI total, mean (SD)*	36.9 (6.2)	34.9 (6.2)	36.1 (6.3)	35.8 (6.6)	31.53 (6.3)	34.1 (6.8)
STAIT, mean (SD)*	47.3 (8.3)	45.9 (6.6)	46.7 (7.6)	46.8 (9.4)	42.05 (8.7)	44.8 (9.4)
CES-DC, mean (SD)	23.9 (11.1)	21.8 (10.3)	23.0 (10.8)	27.0 (12.4)	20.03 (11.9)	24.1 (12.6)
CD-RISC, mean (SD)*	57.3 (18.9)	57.0 (19.7)	57.2 (19.2)	64.7 (17.6)	62.87 (18.8)	64.0 (18.1)
A-COPE, mean (SD)	166.5 (20.3)	166.6 (23.1)	166.6 (21.5)	168.27 (22.3)	166.07 (22.4)	167.4 (22.4)
CTQ Total, median (range)*	43 (25–90)	44 (25–94)	43 (25–94)	40 (25–96)	39 (25–85)	40 (25–96)
AUDIT Total, median (range)*	0 (0–29)	0 (0–28)	0 (0–29)	1 (0–32)	2 (0–27)	2 (0–32)

Abbreviations: CASI, Childhood Anxiety Sensitivity Index; CTQ, Childhood Trauma Questionnaire; STAIT, State-Trait Anxiety Inventory; CES-DC, Center for Epidemiological Studies Depression Scale for children; AUDIT, Alcohol Use Disorders Identification Test; CD-RISC, Connor-Davidson Resilience Scale; A-COPE, Adolescent Coping Orientation for Problem Experiences

There was no significant difference in age between male and female participants for either ethnic group (Xhosa p=0.911 and Coloured p=0.843 respectively). Significant differences between the two ethnic groups with regards to CASI total (p=0. 000005), STAIT total (p=0. 0007), and CD-RISC total (p=0. 0000002) were found. Further non-parametric analysis determined that there was a significant difference between the AUDIT (p=0.002) and CTQ total (p=0.008) scores (Table 3.2). Table 3.3 list the CTQ scores of the participants for each subscale

Table 3.3. Mean and standard deviation of CTQ subscale scores for Xhosa and Coloured participants.

	Xhosa			**Coloured**		
	Male	Female	Both	Male	Female	Both
Physical Neglect	9.44 (SD=3.78)	9.38 (SD=3.76)	9.40 (SD=3.77)	8.05 (SD=3.52)	7.85 (SD=3.16)	7.93 (SD=3.31)
Emotional Abuse	8.9 (SD=3.92)	8.98 (SD=4.07)	8.95 (SD=4.01)	9.61 (SD=4.20)	10.98 (SD=5.05)	10.42 (SD=4.76)
Emotional Neglect	12.33 (SD=5.07)	12.08 (SD=4.94)	12.18 (SD=4.99)	10.78 (SD=4.42)	10.34 (SD=4.46)	10.52 (SD=4.44)
Physical Abuse	8.39 (SD=4.25)	7.83 (SD=4.94)	8.06 (SD=4.1)	7.60 (SD=3.59)	7.59 (SD=4.28)	7.60 (SD=4.01)
Sexual Abuse	7.15 (SD=3.58)	6.73 (SD=3.07)	6.91 (SD=3.29)	6.86 (SD=3.63)	7.12 (SD=4.30)	7.01 (SD=4.03)

The childhood trauma questionnaire has five subscales that determine the CTQ total. Each subscale focuses on a different form of trauma that may be experienced by an adolescent or child. Abbreviation: Standard Deviation (SD)

III.2 *Genotype data*

Genotype data was generated for 19 variants in four different genes. Eighteen variants are SNPs and one, the *5HTTLPR*, is a VNTR polymorphism. Table 3.4 shows the genotype counts for all variants genotyped. With the exception of 5HTTLPR, all the variants were > 90 % successfully genotyped. Genotyping was conducted blind to diagnosis. All the genotyping data was in Hardy-Weinberg equilibrium for both ethnic groups, with the p-value for rejection set at p=0.003 (Table3.4).

Table 3.4 Genotype counts for all variants genotyped.

Variant	Homozygous allele count	%	Heterozygous allele count	%	Homozygous allele count3	%	Total	% of total genotyped
rs3800373(*FKBP5*)	AA = 370	39	AC = 447	47	CC = 135	14	952	97
rs9296158(*FKBP5*)	AA = 188	20	AG = 452	46	GG = 288	31	928	94
rs737054(*FKBP5*)	CC = 805	85	CT = 130	14	TT = 14	1	949	96
rs1360780(*FKBP5*)	AA = 109	12	AG = 432	46	GG = 406	43	947	96
rs6926133(*FKBP5*)	GG = 704	75	TG = 255	27	TT = 15	2	944	96
rs9394309(*FKBP5*)	AA = 663	71	AG = 244	26	GG = 22	2	929	94
rs9470080(*FKBP5*)	CC = 314	33	CT = 467	49	TT = 175	18	956	97
rs11030099(*BDNF*)	AA = 14	1	CA = 146	15	CC = 793	83	953	97
rs6265(*BDNF*)	CC = 874	91	CT = 77	8	TT = 8	1	959	97
rs2049046(*BDNF*)	AA = 101	11	AT = 419	45	TT = 402	44	922	94
rs3813034(*SLC6A4*)	AA = 566	62	AC = 298	33	CC = 42	5	906	92
rs1042173(*SLC6A4*)	AA = 587	61	AC = 319	33	CC = 49	5	955	97
rs6354(*SLC6A4*)	AA = 399	42	AC = 420	44	CC = 126	13	945	96
rs25531(*SLC6A4*)	AA = 676	76	AG = 200	22	GG = 18	2	894	91
rs7209436(*CRHR1*)	AA = 83	9	AG = 366	39	GG = 479	52	928	94
rs4792887(*CRHR1*)	CC = 374	39	CT = 427	45	TT = 156	16	957	97
rs110402(*CRHR1*)	AA = 85	9	AG = 374	40	GG = 478	51	937	95
rs242924(*CRHR1*)	GG = 511	54	TG = 358	38	TT = 85	9	954	97
5HTTLPR	LL = 435	48	LS = 398	44	SS = 74	8	907	92

Totals include the genotype count for all ethnic groups. % of total genotyped expresses the total count of each variant as a percentage of the total number of participants. This table shows the true number of participants genotyped for each variant.

Table 3.5. Hardy-Weinberg Equilibrium p-values and genotype counts for Xhosa and Coloured participants.

Variant	Alleles	Xhosa		Coloured		Variant	Alleles	Xhosa		Coloured	
5HTT		**Counts**	**p-value**	**Counts**	**p-value**	***CRHR1***		**Counts**	**p-value**	**Counts**	**p-value**
rs3813034	AA	432	0.899	126	0.116	**rs7209436**	AA	35	0.499	47	0.490
	CA	153		137			AG	211		149	
	CC	13		24			GG	370		100	
	Total	598		287			Total	616		296	
rs1042173	AA	445	0.657	135	0.165	**rs4792887**	CC	181	0.739	181	0.983
	AC	168		143			CT	310		108	
	CC	18		26			TT	140		16	
	Total	631		304			Total	631		305	
rs6354	AA	221	0.772	163	0.919	**rs110402**	AA	36	0.487	48	0.492
	CA	296		118			AG	214		149	
	CC	104		22			GG	371		98	
	Total	621	0.673	303	0.571		Total	621		295	
rs25531	A/A	433		226		**rs242924**	GG	398	0.714	105	0.792
	A/G	140		58			TG	202		146	
	G/G	13		5			TT	28		54	
	Total	586		289			Total	628		305	
5HTTLPR	L/L	293	0.011	126	0.533						
	L/S	260		126							
	S/S	33		37							
	Total	586		289							

Table 3.5 continued

Variant	Alleles	Xhosa		Coloured		Variant	Alleles	Xhosa		Coloured	
BDNF		**counts**	**p-value**	**counts**	**p-value**	***FKBP5***		**counts**	**p-value**	**counts**	**p-value**
rs11030099	AA	1	0.790	13	0.276	**rs3800373**	AA	225	0.126	134	0.036
	CA	55		85			AC	315		124	
	CC	574		205			CC	85		48	
	Total	630		303			Total	625		306	
rs6265	CC	628	0.968	233	0.446	**rs9296158**	AA	135	0.913	52	0.419
	CT	2		68			AG	311		132	
	TT	0		7			GG	176		102	
	Total	630		308			Total	622		286	
rs2049046	AA	74	0.775	26	0.659	**rs737054**	CC	565	0.080	225	0.154
	AT	284		126			CT	59		67	
	TT	259		135			TT	4		9	
	Total	617		287			Total	628		301	
						rs1360780	AA	70	0.160	38	0.164
							AG	302		122	
							GG	254		140	
							Total	626		300	
						rs9394309	AA	460	0.153	189	0.504
							AG	152		88	
							GG	7		13	
							Total	619		290	
						rs9470080	CC	189	0.561	115	0.435
							CT	317		141	
							TT	121		52	
							Total	627		308	

III.2.1 *Association data*

Of the 18 variants genotyped only nine showed significant associations with CASI total scores (Anxiety sensitivity). Association was first tested across both genders and no statistically significant association observed between genotype and AS in the Xhosa sample, however significance was found in three variants in the Coloured sample. Rs737054 (*FKBP5*) had significance in both the Additive allelic (p=0.04) and Dominant models (p=0.035) in the Coloured population. Rs737054 (*FKBP5*) showed a 1.47 (CI 95%: 0.1-2.84) increase in CASI score assuming a dominant model of inheritance. Rs23351 (*SLC6A4*) was found to be significant with recessive inheritance (p=0.04), however no significance was observed upon further stratification by gender. Furthermore, significant association was observed between *5-HTTLPR* (*SLC6A4*) and AS in the Coloured population (p=0.046) within an additive allelic model of inheritance. Coloured participants show an increase of CASI score of 0.9 for every S allele present (CI 95%: 0.02-1.78). Association was tested further with the Xhosa and Coloured groups stratified by gender. Significance that was observed between *5-HTTLPR* (*SLC6A4*) and CASI total scores with a recessive inheritance in Coloured males (p=0.016). Coloured males with the *SS* genotype had an estimated 3.73 (CI 95%:0.72-6.74) higher mean CASI total score than those Coloured males with *LL* and *LS* genotypes.

Rs3800373 (*FKBP5*) showed significant association with CASI total scores in the Coloured male sample only (p=0.034). Individuals with the heterozygous AC genotype had lower CASI total scores, by an average of 1.96 units (CI 95%: -3.75 to -0.17), than those with the CC genotype. Rs9296158 (*FKBP5*) showed association with CASI total score when assuming a dominant model of inheritance in the Coloured male sample (p=0.007). Coloured males with at least one A allele had an average CASI total score that was 2.66 units (CI 95%: -4.538 to -0.789) lower than those males who had the GG homozygous genotype. Furthermore, rs737054 (*FKBP5*) also showed association with CASI total score in the Coloured male group (p=0.013). This association was found when assuming a dominant model of inheritance, Coloured males possessing at least one T allele had mean CASI total scores that were 2.72 units (CI 95%: 0.66 to 4.78) higher than males with a homozygous CC genotype. In the Xhosa male sample rs6926133 (*FKBP5*) was associated with CASI total score when assuming a recessive model of inheritance (p=0.019). CASI total scores for

Xhosa males homozygous for the T allele were 7.53 units (CI 95%: -13.996 to -1.058) lower than the scores for Xhosa males with at least one C allele.

Rs7209436 (*CHCR1*) showed significant association with CASI total scores in both the Xhosa and Coloured male samples. For Xhosa males in an additive allelic model it was observed that with the addition of an A allele the CASI total score was reduced by 1.49 units (CI 95%: -2.69 to -0.30) (p=0.018). There was a significant association of CASI total score in Coloured males (p=0.02) when a dominant model of inheritance was assumed. Coloured males with at least one A allele had CASI total scores that were, on average, 2.12 units higher than Coloured males that were homozygous for the G allele. Rs110402 (*CHCR1*) also showed significant association with CASI total scores in both the Xhosa and Coloured male groups. In the Xhosa male group, a significant association was observed in an additive allelic model (p=0.023) where the addition of an A allele showed a reduction in CASI total score of 1.47 units (CI 95%: -2.6 to -0.29). Within the Coloured male group there was a significant association assuming a dominant model of inheritance (p=0.045). As the association was not very significant the effect size was not determined. However, Coloured males who possessed at least one A-allele had higher average CASI total scores than those males homozygous for the G allele. Exact p values can be found in Table 3.6 and Table 3.7, significance was taken at $p<0.05$.

Table 3.6 Association of CASI total score and genotype in Xhosa and Coloured population groups using additive allelic, dominant, and recessive models of inheritance without stratification into gender groups.

Variants		Genotype		Additive allelic		Dominant		Recessive	
	Minor allele	Black	Coloured	Black	Coloured	Black	Coloured	Black	Coloured
rs3800373 (*FKBP5*)	C	0.5245	0.5502	0.6787	0.4413	0.3795	0.2965	0.6689	0.9206
rs9296158 (*FKBP5*)	A	0.4821	0.1295	0.4334	0.1710	0.8856	0.0530	0.2377	0.8970
rs737054 (*FKBP5*)	T	0.6929	0.1068	0.7859	**0.0399**	0.6494	**0.0349**	0.5499	0.4192
rs6926133 (*FKBP5*)	T	0.7814	0.7870	0.8518	0.9279	0.7440	0.7704	0.5781	0.5968
rs25531 (*SLC6A4*)	G	0.3277	0.1110	0.1531	0.5210	0.2137	0.9436	0.2649	**0.0404**
5HTTLPR (*SLC6A4*)	S	0.8304	0.1374	0.9921	**0.0462**	0.8125	0.0722	0.6264	0.1557
rs7209436 (*CRHR1*)	A	0.8042	0.6888	0.5951	0.3899	0.7263	0.4327	0.5228	0.5585
rs110402 (*CRHR1*)	A	0.8429	0.9252	0.6872	0.7446	0.8282	0.8691	0.5597	0.6952
rs242924 (*CRHR1*)	T	0.4767	0.7209	0.6429	0.4425	0.4103	0.5967	0.5285	0.4497

Significant p values are shown in boxes. All confounders for association testing were controlled for. Possible confounders were age, gender, depression (CESD total score), resilience (CD-RISC total score), coping mechanisms (A-COPE total score), trait anxiety (STAIT total score), alcohol use disorders (AUDIT total score) and Childhood Trauma (CTQ total score).

Table 3.7 Association of CASI total score with genotype in Xhosa and Coloured populations groups using additive allelic, dominant, and recessive models of inheritance stratified according to gender.

		Genotype				Additive allelic			
Variants		**Xhosa**		**Coloured**		**Xhosa**		**Coloured**	
	Minor allele	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
rs3800373 (*FKBP5*)	C	0.9443	0.4319	0.8998	0.0984	0.9834	0.7027	0.7081	0.041
rs9296158 (*FKBP5*)	A	0.5032	0.9709	0.5852	0.0251	0.5408	0.811	0.9522	0.0112
rs737054 (FKBP5)	T	0.9527	0.3925	0.6217	0.0449	0.801	0.5399	0.3289	0.016
rs6926133 (*FKBP5*)	T	0.2597	0.046	0.7444	0.6936	0.6004	0.8287	0.6043	0.4634
rs25531 (SLC6A4)	G	0.2691	0.7731	0.5943	0.1835	0.1454	0.5255	0.8016	0.5453
5HTTLPR (*SLC6A4*)	S	0.5052	0.3908	0.4129	0.0475	0.3108	0.1964	0.3104	0.0362
rs7209436 (*CRHR1*)	A	0.3943	0.0617	0.8595	0.0602	0.2599	0.0184	0.5837	0.0252
rs110402 (*CRHR1*)	A	0.3982	0.0745	0.5996	0.1168	0.2234	0.023	0.3169	0.0463
rs242924 (*CRHR1*)	T	0.3972	0.1129	0.9817	0.3194	0.1767	0.3115	0.8713	0.13

Significant p values are shown in boxes. MA (minor allele). All confounders for association testing were controlled for. Possible confounders were age, gender, depression (CESD total score), resilience (CD-RISC total score), coping mechanisms (A-COPE total score), trait anxiety (STAIT total score), alcohol use disorders (AUDIT total score) and Childhood Trauma (CTQ total score).

Table 3.7 Continued

		Dominant				Recessive			
Variants		**Xhosa**		**Coloured**		**Xhosa**		**Coloured**	
	Minor allele	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
rs3800373 (*FKBP5*)	C	0.8856	0.3374	0.8318	0.0339	0.8008	0.5867	0.6462	0.2584
rs9296158 (*FKBP5*)	A	0.9791	0.8243	0.6346	0.0072	0.2753	0.8709	0.4898	0.1792
rs737054 (FKBP5)	T	0.8347	0.3559	0.3572	0.0125	0.7891	0.4998	0.5431	0.5503
rs6926133 (*FKBP5*)	T	0.8366	0.7206	0.5017	0.5513	0.1005	0.0193	0.884	0.4716
rs25531 (SLC6A4)	G	0.2081	0.6285	0.9896	0.9782	0.2183	0.5103	0.3156	0.0768
5HTTLPR (*SLC6A4*)	S	0.4758	0.1701	0.1937	0.1913	0.2779	0.7634	0.8568	0.0158
rs7209436 (*CRHR1*)	A	0.1825	0.0313	0.6542	0.02	0.8704	0.1372	0.6561	0.2317
rs110402 (*CRHR1*)	A	0.1758	0.0403	0.3451	0.0453	0.7322	0.1338	0.5251	0.2428
rs242924 (*CRHR1*)	T	0.1882	0.7019	0.9289	0.1956	0.4621	0.0368	0.8486	0.2401

Significant p values are shown in boxes. MA (minor allele). All confounders for association testing were controlled for. Possible confounders were age, gender, depression (CESD total score), resilience (CD-RISC total score), coping mechanisms (A-COPE total score), trait anxiety (STAIT total score), alcohol use disorders (AUDIT total score) and Childhood Trauma (CTQ total score).

III.2.2 *Interaction data*

Interaction between genotype and childhood trauma (CTQ total) was first tested for without stratification by gender. Subsequent investigations were performed in gender-stratified datasets. Rs3800373 (*FKBP5*) showed no interaction between genotype and CTQ total score before gender stratification in the Xhosa and Coloured samples. Nominal interaction between genotype and CTQ total score was seen in the male Coloured population using an additive allelic model of in heritance (p=0.049). In Coloured males for each addition of a C-allele there was a decrease in CASI total of 0.12 units for every unit increase in CTQ total score (CI 95%: -0.24 to 0.00). Rs2049046 (*BDNF*) was found to have significant interaction with CTQ total score in Xhosa females under an additive allelic model (p=0.001). Xhosa females' CASI total decreased by -0.14 units for every unit increase in CTQ total score with the addition of a C-allele (CI 95%: -0.22 to -0.06). Rs1042173 (*SLC6A4*) showed interaction with CTQ total score in the Xhosa sample as a whole (p=0.042). Assuming a recessive model of inheritance there was a 0.315 unit decrease in CASI total for every unit increase in CTQ total score in those Xhosa participants homozygous for the C-allele compared to those with at least one A-allele (CI 95%: -0.611 to -0.019). Rs6354 (*SLC6A4*) also showed no interaction before gender stratification, however was seen to be significant in Xhosa males when considering an additive allelic model of inheritance (p=0.016). In the Xhosa male sample, there was a 0.12 unit decrease in CASI total score for every unit increase in CTQ total score (CI 95%: -0.21 to -0.02) for each *C*-allele present. Exact p values can be found in Table 3.8 and Table 3.9; significance was set at $p<0.05$. Effect sizes and confidence intervals are summarized in Table 3.10.

Table 3.8 Interaction of genotype and CTQ total score with CASI total score using additive allelic, dominant, and recessive models of inheritance not stratified according to gender.

Both genders									
		Genotype		Additive allelic		Dominant		Recessive	
Variant	**MA**	**Xhosa**	**Coloured**	**Xhosa**	**Coloured**	**Xhosa**	**Coloured**	**Xhosa**	**Coloured**
rs3800373 (*FKBP5*)	C	0.8175	0.9694	0.9647	0.7558	0.6671	0.8110	0.8155	0.8515
rs2049046 (*BDNF*)	A	0.1150	0.0934	0.1169	0.6211	0.0443	0.6066	0.9958	0.0733
rs1042173 (*SLC6A4*)	C	0.0581	0.8805	0.0424	0.6266	0.1099	0.7137	0.2513	0.8762
rs6354 (*SLC6A4*)	C	0.2259	0.9120	0.4100	0.7973	0.9632	0.9348	0.2937	0.5001

Significant p values are shown in boxes. MA (minor allele). All confounders for association testing were controlled for. Possible confounders were age, gender, depression (CESD total score), resilience (CD-RISC total score), coping mechanisms (A-COPE total score), trait anxiety (STAIT total score), and alcohol use disorders (AUDIT total score).

Table 3.9 Interaction of genotype and CTQ total score with CASI total score using additive allelic, dominant, and recessive models of inheritance stratified according to gender.

		Genotype				Additive allelic			
		Xhosa		Coloured		Xhosa		Coloured	
Variant	MA	female	male	female	male	female	male	female	male
rs3800373 (*FKBP5*)	C	0.3698	0.1956	0.8488	0.4074	0.4006	0.2773	0.6770	0.0486
rs2049046 (*BDNF*)	A	0.0058	0.0570	0.4556	0.3721	0.0010	0.0934	0.2043	0.3142
rs1042173 (*SLC6A4*)	C	0.6293	0.8963	0.9147	0.4909	0.4923	0.2734	0.5449	0.3539
rs6354 (*SLC6A4*)	C	0.2416	0.0353	0.9859	0.8860	0.1845	0.0159	0.9753	0.6732

		Dominant				Recessive			
		Xhosa		Coloured		Xhosa		Coloured	
Variant	MA	female	male	Female	male	female	male	female	male
rs3800373 (*FKBP5*)	C	0.7684	0.3707	0.7351	0.1041	0.1561	0.1047	0.6967	0.2104
rs2049046 (*BDNF*)	A	0.0015	0.4373	0.6188	0.1824	0.0863	0.0181	0.2219	0.8604
rs1042173 (*SLC6A4*)	C	0.5442	0.4094	0.9762	0.2632	0.4591	0.7471	0.6464	0.6258
rs6354 (*SLC6A4*)	C	0.1043	0.0721	0.9430	0.7106	0.7066	0.0176	0.8578	0.9102

Significant p values are shown in boxes. MA (minor allele). All confounders for association testing were controlled for. Possible confounders were age, gender, depression (CESD total score), resilience (CD-RISC total score), coping mechanisms (A-COPE total score), trait anxiety (STAIT total score), and alcohol use disorders (AUDIT total score).

Table 3.10 Variant Interaction with CTQ showing effect sizes and confidence intervals.

Variant	Sample Group	Inheritance	Comparison		Effect	Pr(>|t|)	95% CI	
rs3800373 (*FKBP5*)	Coloured males	Additive allelic	Added C-allele		-0.12	0.049	-0.24	0
rs2049046 (*BDNF*)	Xhosa females	Additive allelic	Added A-allele		-0.14	0.001	-0.22	-0.06
rs1042173 (*SLC6A4*)	Xhosa all	Recessive	CC	AA+AC	-0.315	0.037	-0.611	-0.019
rs6354 (*SLC6A4*)	Xhosa males	Additive allelic	Added C-allele		-0.12	0.016	-0.21	-0.02

All confounders for association testing were controlled for. Possible confounders were age, gender, depression (CESD total score), resilience (CD-RISC total score), coping mechanisms (A-COPE total score), trait anxiety (STAIT total score), and alcohol use disorders (AUDIT total score).

III.3 *Haplotype analysis*

Haplotype analysis was performed using the Linkage disequilibrium (LD) package in RStudio for all the genotyped variables and were confirmed using Haploview for those variables that were SNPs. In Haploview LD plots with D' values according to Gabriel et al.,2002 recommended CI criteria (default Haploview settings) were created (Figure3.1 and Figure 3.2).

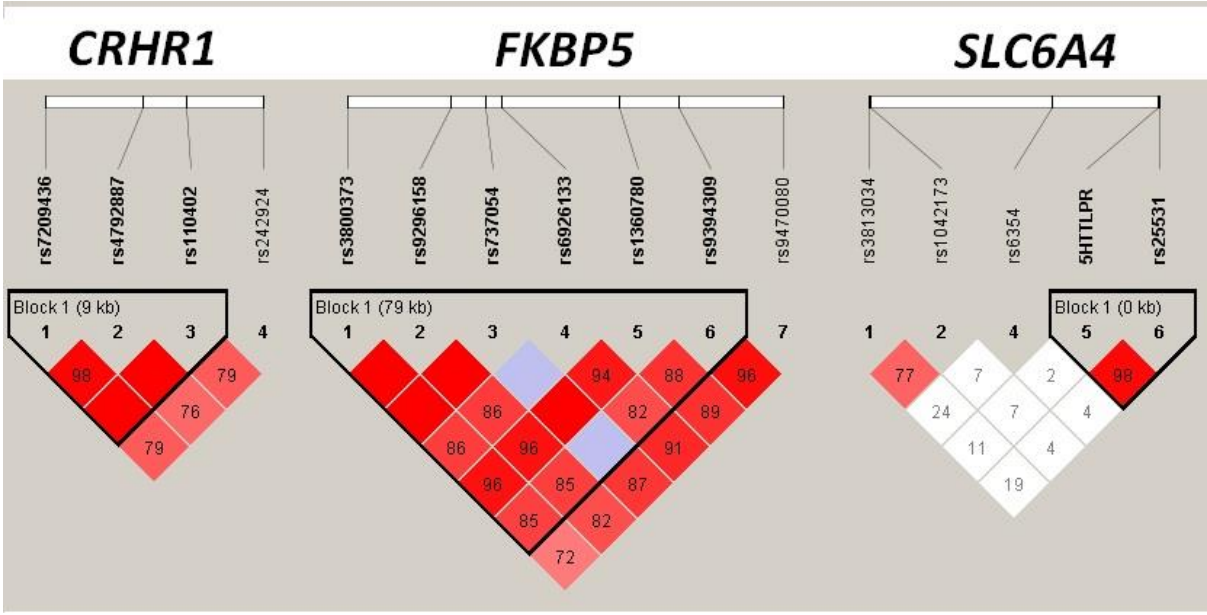

Figure 3.1 Linkage disequilibrium map for the Xhosa participants, D' values are depicted in the diamonds, with darker red depicting stronger LD. The LD map was created using the confidence intervals (CI), implemented in Haploview (Gabriel et al. 2002).

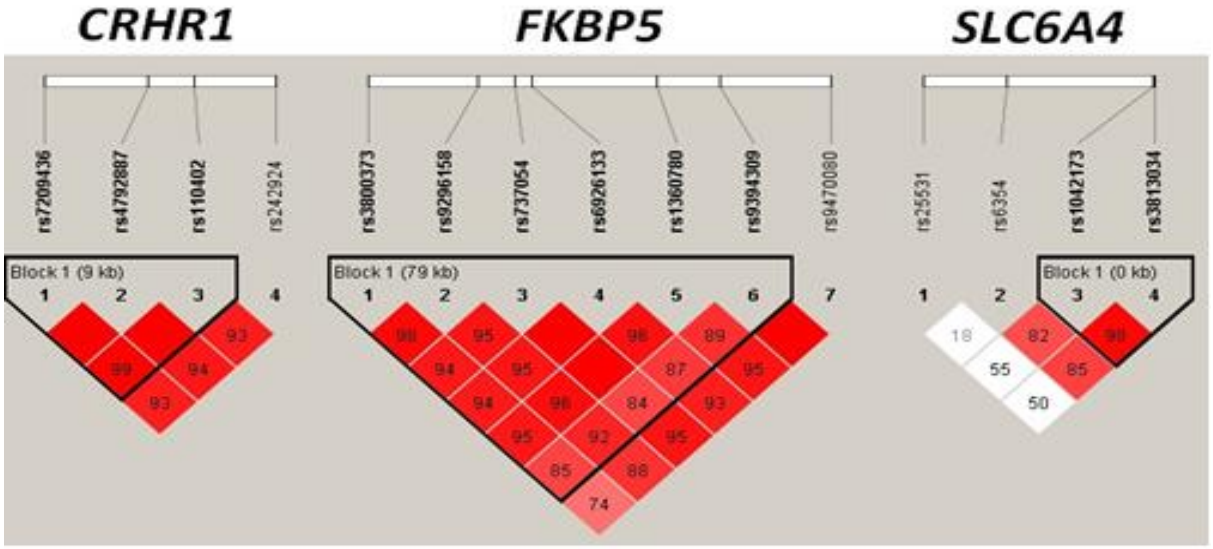

Figure 3.2 Linkage disequilibrium map for the Coloured participants, D' values are depicted in the diamonds, with darker red depicting stronger LD. The LD map was created using the confidence intervals (CI), implemented in Haploview (Gabriel et al. 2002).

Table 3.11 Haploblocks observed in Xhosa and Coloured sample groups as determined using confidence intervals.

Haploblock	**Gene**	**Ethnicity**
rs25531 and *5HTTLPR*	*SLC6A4*	Xhosa
rs3813034 and rs1042173	*SLC6A4*	Coloured
rs7209436, rs4792887 and rs110402	*CRHR1*	Xhosa and Coloured
rs3800373, rs9296158, rs737054, rs6926133, rs1360780, and rs9394309	*FKBP5*	Xhosa and Coloured

Association with anxiety and interaction of CTQ and anxiety of the different haploblocks was tested for within Xhosa and Coloured samples. There was only one significant association with anxiety in the Xhosa sample when both genders were considered together (Table3.12). Significance was observed between the rs25531 and *5HTTLPR* haplotype and AS in the Xhosa sample (p=0.021). Xhosa participants who carried the *G-L* haplotype showed an estimated decrease in CASI total score of 1.29 for each copy of the *G-L* haplotype compared to the reference *A-L* haplotype (p=0.010) (Table 3.13).

Table 3.12 Association of Haploblocks with anxiety and Interaction between Haploblocks and CTQ with Anxiety for Xhosa and Coloured participants without gender stratification.

Haploblock	**Both genders**			
	Association		**Interaction**	
	Xhosa	**Coloured**	**Xhosa**	**Coloured**
rs25531 and *5HTTLPR* (*SLC6A4*)	0.021		0.059	
rs3813034 and rs1042173 (*SLC6A4*)		0.414		0.733
rs7209436, rs4792887 and rs110402 (*CRHR1*)	0.771	0.354	0.115	0.478
rs3800373, rs9296158, rs737054, rs6926133, rs1360780, and rs9394309 (*FKBP5*)	0.347	0.638	0.353	0.592

Table 3.13 rs25531 and*5HTTLPR* haplotype association with AS in the Xhosa sample. Effect is estimated in the difference between specific haplotype and reference haplotype. Significance was taken at $p < 0.05$.

rs25531 and 5HTTLPR Haplotype association with AS						
				95% CI		
rs25531	*5HTTLPR*	**hap.freq**	**Effect**	**Lower**	**Upper**	**p-value**
A	S	0.29	-0.308	-1.05	0.44	0.419
G	L	0.12	-1.293	-2.28	-0.31	0.01
G	S	0.01	2.228	-1.4	5.85	0.229
A	L	0.58	Reference haplotype			

No further association was found when the Xhosa sample was stratified by gender for the rs25531 and *5HTTLPR* haplotype (males: p=0.177) and females: p=0.071) (Table 3.14).

Table 3.14 Association of Haploblocks with anxiety for Xhosa and Coloured participants with gender stratification. All confounders for association testing were controlled for. Possible confounders were age, gender, depression (CESD total score), resilience (CD-RISC total score), coping mechanisms (A-COPE total score), trait anxiety (STAIT total score), alcohol use disorders (AUDIT total score) and Childhood Trauma (CTQ total score).

Haploblock	**Association with AS**			
	Xhosa		**Coloured**	
	Female	**Male**	**Female**	**Male**
rs25531 and *5HTTLPR* (*SLC6A4*)	0.070	0.177		
rs3813034 and rs1042173 (*SLC6A4*)			0.553	0.982
rs7209436, rs4792887 and rs110402 (*CRHR1*)	0.192	0.341	0.905	0.026
rs3800373, rs9296158, rs737054, rs6926133, rs1360780, and rs9394309 (*FKBP5*)	0.181	0.819	0.635	0.884

When participants were stratified by gender there was an association between the rs7209436, rs4792887 and rs110402 (*CRHR1*) haplotype and AS in Coloured males only (p=0.026). Coloured males carrying the *A-C-A* haplotype showed an estimated average increase of CASI total score of 2.29 units for each additional copy of the *A-C-A* haplotype compared to the reference *G-T-G* haplotype (p=0.004).

No interaction between haplotype and CTQ total score and AS was observed when the Xhosa and Coloured sample groups were analysed in total (without gender stratification)(Table 3.12). However, significant interaction was observed in the Xhosa female participants and the rs25531 and *5HTTLPR* (*SLC6A4*) haplotype (p=0.014). Xhosa females who possessed the *G-L* haplotype showed a 0.13 unit reduction in CASI total score for every unit increase in CTQ total score (p=0.003) compared to those females with the *A-L* haplotype (Table 3.16).

Table 3.15 Interaction between haplotype and CTQ total score with AS in Xhosa and Coloured participants, stratified by gender.

Haploblock	**Interaction with CTQ on anxiety**			
	Xhosa		**Coloured**	
	Female	Male	**Female**	Male
rs25531 and *5HTTLPR* (*SLC6A4*)	0.014	0.904		
rs3813034 and rs1042173 (*SLC6A4*)			0.234	0.603
rs7209436, rs4792887 and rs110402 (*CRHR1*)	0.472	0.422	0.521	0.857
rs3800373, rs9296158, rs737054, rs6926133, rs1360780, and rs9394309 (*FKBP5*)	0.925	0.069	0.775	0.034

Table 3.16 rs25531 and*5HTTLPR* haplotype interaction with CTQ total score on AS in the Xhosa female sample. Effect is estimated in the difference between specific haplotype and reference haplotype. Significance was taken at $p < 0.05$.

rs25531 and 5HTTLPR Haplotype interaction with CTQ total score on AS

rs25531	*5HTTLPR*	hap.freq	Effect	se	p-value
A	**S**	0.28	-0.07	0.04	0.071
G	**L**	0.13	-0.13	0.05	0.003
G	**S**	0.01	-0.16	0.12	0.204
A	**L**	0.58	Reference haplotype		

No significant association was observed between the *FKBP5* haploblock (rs3800373, rs9296158, rs737054, rs6926133, rs1360780, and rs9394309 (*FKBP5*)) and AS, however there was an interaction with CTQ on anxiety in the Coloured male sample (p=0.034) (Table 3.15). CASI total scores (representing AS) were found to increase, on average, by 0.43 units for every one unit increase in CTQ total score (representing level of childhood trauma) in Coloured males with the haplotype *A-G-C-T-A-A*, relative to Coloured males with the *A-G-C-G-G-A* haplotype (p=0.007) (Table 3.17). Coloured males who carried the C-A-C-T-A-G haplotype showed an estimated average decrease in CASI total scores of 0.22 (p=0.012) (Table 3.17), compared to the reference *A-G-C-G-G-A* haplotype. Rare haplotypes were combined and no significant effects were found.

Table 3.17 rs3800373, rs9296158, rs737054, rs6926133, rs1360780, and rs9394309 (*FKBP5*) haplotype interaction with CTQ total score on AS in the coloured male sample. Haplotypes were tested individually after which rare haplotypes were combined (haplotype frequency < 0.01).

***FKBP5* haplotype interaction with CTQ on AS**										
	rs3800373	**rs9296158**	**rs737054**	**rs6926133**	**rs1360780**	**rs9394309**	**hap.freq**	**coef**	**se**	**pval**
haplotype 1	A	A	C	G	G	A	0.04	0.01	0.13	0.906
haplotype 6	A	G	C	T	A	A	0.01	0.43	0.16	0.007
haplotype 7	A	G	T	G	G	A	0.06	-0.19	0.1	0.069
haplotype 8	C	A	C	G	A	A	0.18	-0.05	0.08	0.516
haplotype 9	C	A	C	G	A	G	0.04	0.01	0.2	0.958
haplotype 10	C	A	C	G	G	A	0.04	-0.05	0.11	0.668
haplotype 12	C	A	C	T	A	G	0.14	-0.22	0.08	0.012
rare haplotype	*	*	*	*	*	*	0.01	-0.58	0.51	0.257
	A	**G**	**C**	**G**	**G**	**A**	**0.49**	**Reference haplotype**		

Chapter 4

Discussion

The present study examined genetic and clinical data, consisting of anxiety sensitivity and childhood trauma, obtained from Xhosa and Coloured South African adolescents to determine whether AS can be utilized as a predictive tool to determine early risk susceptibility for anxiety disorder diagnoses.

IV.1 Variants with in FKBP5, SLC6A4, CRHR1 and BDNF may serve as potential early detection biomarkers for susceptibility risk for anxiety disorders

IV.1.1 FKBP5

Association between AS and four SNPs in *FKBP5* were found to be significant in the Coloured sample. Firstly, there was a significant association between AS and rs737054 in the Coloured group as a whole (Table 3.7). This significance persisted in Coloured males (p=0.016), but was not seen in females (p=0.329). Why rs737054 is significant in Coloured males only is unclear. However, cortisol level differences between males and females in the context of anxiety have been described in literature before (Kudielka et al., 2004; Schiefelbein and Susman, 2006). Although the precise functional implications of rs737054 are not known, this variant could play a role in the regulation of cortisol secretion, as *FKBP5* has been linked to cortisol level changes in response to stressors (Buchmann AF et al., 2014; Mahon et al., 2013a). More specifically *FKBP5* has been found to play a role in emotion processing via amygdala activity (Holz et al., 2015), which could allude to a possible mechanism by which variation in *FKBP5* could confer susceptibility to anxiety and related disorders. Despite a lack of knowledge concerning the functional role of rs737054, it is important to note that this variant is located within a highly conserved region of intron 5 which has been previously reported to have regulatory potential (King et al., 2005; Willour et al., 2009). Further studies are required to fully characterize the extent to which the variant may influence AS as well as anxiety and anxiety-related disorders.

When stratifying for gender, the rs3800373 and rs9296158 were both found to be significantly associated with AS under a dominant model of inheritance (Table 3.7). rs3800373 showed an average decrease in AS in Coloured males who were heterozygous (*AC*) compared to Coloured males who were homozygous for the *C* allele. This variant has been studied extensively with regard to cortisol levels in response to stress (Comasco et al., 2015; Ising et al., 2008; Mahon et al., 2013a) as well as its interaction with childhood trauma

(Binder et al., 2008; Buchmann AF et al., 2014; Luijk et al., 2010). The *CC* genotype has been associated with higher levels of cortisol in response to stress and dysregulation of the HPA axis (Binder et al., 2004b; Collip et al., 2013; Ising et al., 2008; Luijk et al., 2010). Our findings support where a heterozygous advantage was observed. *AC* individuals showed a higher relative fitness compared to *CC* homozygotes, which is in line with previous findings. Furthermore, the GxE effect observed in our study (Table 3.9) is congruent with previous studies where the modulatory effect of childhood trauma was limited to males only, and is seen to be desensitizing (Comasco et al., 2015).

In this study the rs9296158 was found to be associated with low AS scores in individuals possessing at least one *A* allele. Unlike rs3800373, this finding contradicts previously reported literature(Collip et al., 2013; White et al., 2012; Zimmermann P et al., 2011), where the *A* allele was found to confer increased risk for post-traumatic stress disorder (PTSD)(Binder et al., 2008). It may be that this variant, while predisposing individuals to risk for the development of PSTD, may infer protection in the South African cohort. Considering interaction, in haplotype analyses CTQ modified the relationship between AS and variants within *FKBP5*. Haplotype 12 (coef = -0.22; Table3.16) was found to be significant in the Coloured male sample (p=0.012) and reduced AS scores as the severity of childhood trauma increased. This haplotype was not the most significant finding in this analysis, however both minor alleles (*C* allele, rs3800373 and *A* allele, rs9296158) were present in that haplotype. This could mean that the desensitizing effect discussed for rs3008373 and rs9296158 above are collectively responsible for the reduced susceptibility to AS observed. This theory is strengthened by the opposing directional effect observed when both major alleles are present, respectively (haplotype 6, coef = 0.43; Table 3.17).

As shown above, the significant associations observed for variants within *FKBP5* have previously been reported to be associated with a risk for developing anxiety- or anxiety-related disorders. The association of these same variants with AS independent of an anxiety disorder diagnosis flags these variants as potential early predictive biomarkers that may be used in a clinical setting for the early assessment of risk to develop anxiety disorders. That said, it is important to note that significant findings were isolated to a single ethnic group and gender. Validation of the findings presented here in a replication cohort, as well and characterization in other ethnic groups, is warranted.

IV.1.2 SLC6A4

The *5HTTLPR* is a region in *SLC6A4* that has been extensively studied in relation to psychiatric disorders such as depression (Ho et al., 2013; Kendler et al., 2005; Power et al., 2010b; Sharpley et al., 2014; Van der Auwera et al., 2014), panic disorder, bipolar disorder, affective disorders, and other psychiatric symptoms (Gyawali et al., 2010; Jorm et al., 1998b; Laucht et al., 2009a; Tharoor et al., 2013). It has also been studied in relation to anxiety disorders and AS (Klauke et al., 2011c; Laucht et al., 2009a; Stein et al., 2007b). In this study, the *S* allele was found to be associated with increased AS scores in the Coloured population (p=0.046) (Table3.5). This is in line with literature which has reported the *S* allele as a risk allele for the development of depression (Haenisch et al., 2013), as well as linked to higher levels of AS (Zavos et al., 2012b). This increase in AS was even more pronounced in Coloured males homozygous for the *S* allele compared to those who were heterozygous, or homozygous for the *L* allele (effect size = 3.37) (section 3.2.1). This, again, supports what is seen in literature (Haenisch et al., 2013). Neuroimaging studies have found that carriers of the *S* allele show increased amygdala activation compared to *LL* genotype carriers, which has been suggested as a possible mechanism for susceptibility risk to anxiety disorders (El-Hage et al., 2013).

Rs25531 is a SNP that is included in most studies looking at *5HTTLPR* as it has been reported to modify the expression of the *LL* genotype in that the presence of a *G* allele (L_g) has been associated with decreased serotonin reuptake, allowing for a phenotype similar to that of the *S* allele (Hariri and Holmes, 2006; Oathes et al., 2015; Parsey et al., 2006). Haplotype analysis of rs25531 and *5HTTLPR* yielded a decrease in AS in the Xhosa sample (Table 3.13). When stratifying for gender, childhood trauma was found to modify AS scores in Xhosa females for the same haplotype, presented as a 0.13 AS total score unit decrease in AS for every unit increase in childhood trauma (Table 3.16). These effects are in contrast with the above mentioned literature where the expected effect of the L_g allele, which is associated with increased levels of anxiety sensitivity, especially in the presence of adverse life events (Oathes et al., 2015; Stein et al., 2007b) is not seen in our study.

The relationship between two additional variants and AS were found to be modified by with increasing severity of childhood trauma. The first, rs1042173 in the Xhosa sample as a whole (Table 3.8) and the second, rs6354 in Xhosa males only. Rs1042173 is often studied in the

context of substance dependence and addiction (Demers et al., 2014; Johnson et al., 2011; Kenna GA et al., 2012; Seneviratne et al., 2013, 2009) as well as in suicidality studies (El-Sayed et al., 2012; Enoch et al., 2013; Pinto et al., 2011). There are few studies that have definitively linked this variant to changes in anxiety, however rs1042173 is found in the 3' untranslated region (3'-UTR), which could account for a translational modification leading to decrease in anxiety sensitivity in the presence of trauma. Rs1042173 has been found to disrupt certain miRNA binding, more specifically miRNA 135 ("miRdSNP," n.d.), which has been linked to alterations in anxiety and depression within mouse models (Issler et al., 2014). Furthermore levels of miRNA 135 were found to be significantly lower in patients with depression and miRNA have been linked in literature to adaptive responses to stress and anxiety (Haramati et al., 2011; Issler et al., 2014). Since rs1042173 possibly disrupts miRNA binding we would expect to see an increase in AS as the proposed function of miRNA 135 would be as an endogenous antidepressant/anxiolytic (Issler et al., 2014). In our cohort the interaction with CTQ shows a decrease in AS, which lend credence to desensitizing effect of childhood trauma

In Xhosa males rs6354 was associated with decreased AS scores as the severity of childhood trauma increased (Table 3.9). This variant is found in the 5' untranslated region (5'-UTR), postulating the modified relationship to AS may be due to potential regulatory mechanisms. There are no published studies indicating that this finding is limited to males only. Inconsistency in findings across studies has been linked to variation in age of participants included in those studies (Uddin et al., 2010).

Rs6354 has been linked to depression and anxiety in other studies (Ho et al., 2013; Liu et al., 2010; Su et al., 2009; Wray et al., 2009) with the *A* allele in particular having been found to be associated with increased risk for anxiety and depression (Wray et al., 2009). It is therefore plausible that the variants found to be significant in this study, and previously reported to be associated with risk of developing anxiety disorders, may be potential biomarkers when used in conjunction with AS clinical assessments to identify individuals at risk for developing anxiety disorders.

IV.1.3 CRHR1

There is evidence for the involvement of *CRHR1* in mood and anxiety disorders (Holsboer and Ising, 2010; Klengel et al., 2013b; Spijker and Van Rossum, 2012). Previous studies have shown that a haplotype (rs7209436-rs110402-rs242924; *A-A-T*) increased the risk for stress-related disorders, such as PTSD and depression (Bradley RG et al., 2008; White et al., 2013). In this study, rs7209436 *A*-allele was associated with increased AS in Coloured males. The directional effect, however, was reversed in Xhosa males (Section 3.2.1; Table 3.7). Furthermore, rs110402 presented significant associations in both Xhosa and Coloured male sample groups.

Haplotype analyses identified a single significant haplotype block (rs7209436-rs4792887-rs110402; *A-C-A*) in our Coloured, male cohort (Table 3.14). Coloured males who had the *A-C-A* haplotype were found to have a higher AS. This finding is concordant with the aforementioned literature. Furthermore, these findings support observations of gender specific effects for variation in genes involved in steroid hormones (as seen with *FKBP5* above (Mahon et al., 2013b)). Sex differences in stress responses has been shown in literature as well as differences in amygdala and hippocampus function (Pagliaccio et al., 2015). This suggests that the male effects seen in our cohort may be linked to cortisol regulation and function in response to stress and traumatic life events.

IV.2 *Limitations*

IV.2.1 *Cohort size and ethnic factors*

The sample size of this study may be considered reasonable; however, the loss of participants, whether it was from sampling error or wet bench error is a limitation. A confounding factor may be population stratification. Participants were asked to self-classify themselves, of which the largest ethnic groups were Xhosa followed by Coloured. The Xhosa ethnic group consisted of mainly isiXhosa speaking individuals which is the second largest ethnic group in South Africa ("South Africa's population," n.d.). Although there is no specific data related to the underlying genetic substructure in the Xhosa population, it has been found that there is relative genetic homogeneity within the population group (Bryc et al., 2010; Niehaus et al., 2005; Tishkoff et al., 2009; Veeramah et al., 2012). In contrast to this, the South African Coloured population comprises Khoesan, Bantu-speaking, European and

Asian ancestry ("South Africa's population," n.d.). This results in a high level of genetic heterogeneity which could account for some of the discrepancies found in this study.

IV.2.2 *Clinical data*

All of the screening tests employed to determine the clinical variables used in this study were gathered using retrospective and self-report methods and may be subject to some biases such as recall bias. Although some of the testing methods have built in methods to correct for certain biases it is always prudent to keep them in mind as possible explanations for discrepancies or outlier data. The South African population presents a unique problem when it comes to any study involving complex disorders with multifactorial aetiology. The population distribution makes any sort of generalization of findings untenable (Seedat et al., 2008) not only from a psychopathological view, but also from a genetic perspective. For this reason, the findings reported in the previous section were necessarily distributed into Xhosa and Coloured population groups.

IV.2.3 *Childhood trauma questionnaire*

The CTQ consists of five subscales: physical neglect, emotional abuse, emotional neglect, physical abuse, and sexual abuse. Some of the subscales have been reported to be more associated with anxiety disorders. For the purposes of this study CTQ total scores were considered, however using each individual subscale may have yielded different associations and interactions.

IV.2.4 *Polymorphism selection*

A TagSNP approach was used to identify specific SNPs within the genes of interest. This approach is flawed in that the population data from www.hapmap.org does not necessarily correlate with the South African population. To account for this builds from different population groups were used to identify selected SNPs.

IV.2.5 *Statistical analysis*

The exploratory nature of the study placed some limitations on the rigour of the statistical analysis. No correction for multiple testing was applied to avoid the chance of excluding certain associations.

IV.2.6 *BDNF*

BDNF is well studied in relation to depression and anxiety, however in our cohort there were difficulties in performing statistical analysis on our data. This was due to the fact that there were low counts of certain genotypes in both the Xhosa and Coloured participants (Table3.4).

IV.3 *Future studies*

Findings in the *FKBP5* gene in our study were limited to Coloured males only, similarly findings in *CRHR1* were also limited to one sex. These findings may point to gender specific variations in genes regulating steroid hormone function, which could be an area that future studies could examine. Our findings also implicate HPA axis dysregulation and possible involvement of certain brain regions such as the amygdala in the aetiology of anxiety disorders. Further study into the functionality of certain SNPs such as rs737054 (*FKBP5*) and others may also bring new insights to the mechanisms underlying susceptibility risk to anxiety disorders manifesting in adolescence and adulthood.

Future studies should use the CTQ subscales in conjunction with CTQ total scores. Some of the subscales such as emotional neglect and abuse have been found to be associated with greater symptom severity in social anxiety disorder (Simon et al., 2009). Using the subscales of CTQ may lend more specificity to what the underlying mechanisms of action may be.

Conclusion

This study aimed to characterize genetic variation previously reported to be involved in anxiety- and anxiety—related disorders as potential early predictors in susceptibility for disease. In this study we presented significant findings for variants within *SLC6A4*, *BDNF*, *CRHR1*, and *FKBP5* and AS. AS may be seen as a precursor to the development of anxiety- and anxiety-related disorders, and therefore a potential clinical marker facilitating early diagnoses. The identification of significant associations between AS and variants previously associated with risk for an anxiety disorders highlights these as potential candidate molecular markers for facilitating early diagnosis in conjunction with the currently available clinical tools. Furthermore, this study identified significant modifying effects of early adversity on anxiety-related phenotypes, in this case AS. Overall our findings show that increased severity of childhood adversity served to desensitize individuals to the anticipation of anxiety-associated symptoms. Whether this hypothesized desensitization serves as protective against the development of anxiety disorders, contributes to the pathogenicity of these conditions, or both depending on the specific disorder, requires further investigation.

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