The role of DNA methylation in the aetiology of Anxiety Proneness in a cohort of South African adolescents: An exploratory study

by

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Thesis presented in partial fulfilment of the requirements for the degree Master of Science (Human Genetics) in the faculty of Medicine and Health Sciences Stellenbosch University

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March 2017

Declaration

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ACKNOWLEDGEMENTS

I would like to acknowledge all the individuals, groups and institutions for their input in the study and the help provided to me during the course of the past two years.

Thank you to all individuals in the Department of Molecular Biology and Human Genetics as well as the SArchi Psychiatry group and the Magic Lab. Special thanks to all individuals within the Neuropsychiatric Lab team. Thank you to all staff and students for providing me with a pleasant work environment where one can't help but feel the motivation (and some pressure). Thanks for exposing me to various fields of science as well and broadening my scope and definition of "good" science.

Many thanks to my supervisor Dr Sian Hemmings, as well as my self-appointed co-supervisor, Dr. Stefanie Malan-Muller, for granting me the opportunity to be your student. Thank you for your supervision, guidance and support in the duration of this study. Thank you for always making time for me even when you're extremely busy as well.

Thank you to the National Research Foundation (NRF) and the HW Truter Bursary Foundation for their financial assistance during the course of the study.

To all my family and friends, thank you for all your love and support over the past two years.

ABSTRACT

Anxiety disorders are a range of complex disorders characterised by abnormal anxiety in the absence of anxiety-provoking stimuli. The aetiology of anxiety disorders is attributed to interaction between genetic and environmental factors. It is crucial to identify and characterise risk factors that result in the development of anxiety disorders in adulthood. Anxiety proneness (AP) is one such risk factor and represents an endophenotype for anxiety disorders. Several studies have previously associated hypothalamic- pituitary- adrenal (HPA) axis related genes with anxiety disorder development, however these studies generally do not account for environmental contributors. Epigenetics provides a mechanism to study environment and gene interaction. DNA methylation is a well-studied epigenetic modification with the ability to influence gene expression. The present study aimed to investigate the role that genome-wide alterations in DNA methylation play in the development of AP by investigating South African adolescents who have experienced differing levels of childhood trauma (n = 63). The Illumina HumanMethylation450K BeadChip array was used to conduct genome-wide DNA methylation analysis on 46 individuals. EpiTYPER MassARRAY technology was used to verify selected differentially methylated genes from the initial analysis, as well as to analyse additional HPA-axis related genes. Several genes were found to be differentially methylated prior to correction for multiple testing, and the majority of these had designated brain functions. Additionally, a significant trend in methylation was observed in the promoter region of the serotonin transporter gene (*SLC6A4*). In conclusion, the current study indicates a role for the methylation of *SLC6A4* in the development of AP in South African adolescents, however these results need to be replicated in a larger study sample.

OPSOMMING

Angsversteurings beskryf 'n verskeidenheid van komplekse siektes wat gekenmerk word deur abnormale angs in die afwesigheid van angs uitdagende stimuli. Die etiologie van angsversteurings word toegeskryf aan die interaksie tussen genetiese- en omgewings faktore. Daarom is dit noodsaaklik om risiko faktore wat lei tot die ontwikkeling van volwasse angsversteurings te identifiseer. Angs geneigheid verteenwoordig een van hierdie sogenoemde risiko faktore. Angs geneigheid verteenwoordig ‘n angsversteurings endofenotipe wat in die huidige studie ondersoek word. Verskeie studies het voorheen hipotalamus-pituïtêre-adrenale siklus (HPA) verwante gene met die ontwikkeling van angsversteurings assosieer. Oor die algemeen, neem hierdie studies egter nie die bydrae van omgewings faktore in ag tydens analise nie. Epigenetika bied 'n metode om die interaksie tussen genetiese en omgewings faktore te bestudeer. DNS-metilering is 'n goed-bestudeerde epigenetiese modifikasie met die vermoë om geenuitdrukking te beïnvloed. Die huidige studie het ten doel om die rol wat genoom-wye veranderinge in DNS-metilering in die ontwikkeling van AP speel te ondersoek. Hierdie doel was bereik deur Suid-Afrikaanse tieners wat verskeie vlakke van trauma ervaar het tydens hul kinderjare, te ondersoek (N = 63). Die Illumina HumanMethylation450K BeadChip tegnologie is gebruik om genoom-wye DNS-metilering analise uit te voer op 46 individuele. Daarbenewens is die EpiTYPER MassARRAY tegnologie gebruik om selektiewe differensieël gemetileerde gene vanaf die aanvanklike ontleding te verifieer asook bykomende HPA-verwante gene te analiseer. Verskeie differensieel gemetileerde gene was identifiseer voor statistiese korreksie vir veelvuldige toetsing. Die meerderheid hiervan besit toegeskrewe breinfunksies. Daarbenewens was 'n beduidende neiging in metilering ook waargeneem in die promotor streek van die serotonien karweier geen (*SLC6A4*). Ten slotte, dui die huidige studie op 'n moontlike rol vir die metilering van *SLC6A4* in die ontwikkeling van AP in Suid-Afrikaanse tieners. Hierdie resultate moet egter herhaal word in 'n groter studie monster.

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List of Abbreviations

5’	Five Prime End
5-HTTLPR	Serotonin Transporter-Linked Polymorphic Region
5mC	Five-Methylcytosine
3’	Three Prime End
ACE	Angiotensin-Converting Enzyme
ADCYAP1	Adenylate Cyclase Activating Polypeptide 1
ADCYAP1R1	ADCYAP1 Receptor Type 1
AP	Anxiety Proneness
AS	Anxiety Sensitivity
BAI2	Brain-Specific Angiogenesis Inhibitor 2
BDNF	Brain-Derived Neurotrophic Factor
bp	Base Pairs
CASI	The Childhood Anxiety Sensitivity Index
CBT	Cognitive Behavioural Therapy
Chr	Chromosome
CRH	Coricotropin Releasing Hormone
CNS	Central Nervous System
COMT	Catechol-O-Methyltransferase
CpG	Cytosine-Guanine Dinucleotide
CTQ	Childhood Trauma Questionnaire
DMNT	DNA Methyltransferase
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
DSM-V	Diagnostic Manual of Mental Disorders, Fifth Edition
ELS	Early Life Stress
EWAS	Epigenome-Wide Association Study

FKBP5	FK506 Binding Protein
GAD	Generalized Anxiety Disorder
GAD1	Glutamate Decarboxylase 1
GALNT9	N-acetyl Galactosaminyl Transferase 9
GABRP	Gamma-Aminobutyric Acid Type A Receptor Pi Subunit
GWAS	Genome Wide Association Study
HLA-DQB1	Major Histocompatibility Complex, DQ Beta 1
HLA-DRB1	Major Histocompatibility Complex, DR Beta 1
HPA	Hypothalamic Pituitary Adrenal
ID	Identity
IGF	Insulin-like Growth Factor
IL	Interleukin
IPA	Ingenuity Pathway Analysis
LINE1	Long Interspersed Nuclear Element 1
MAN2C1	Mannosidase Alpha Class 2C Member 1
MAOA	Monoamine-Oxidase A
MDD	Major Depressive Disorder
Met	Methionine
mRNA	Messenger RNA
n	Number
NAD	Nicotinamide Adenine Dinucleotide
nl	Nanoliter
NPSR1	Neuropeptide S Receptor 1
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1
OXTR	Oxytocin Receptor
PBMC	Peripheral Blood mononuclear cells
PCR	Polymerase Chain Reaction
PFC	Pre-Frontal Cortex
PRKCZ	Protein Kinase C Zeta

PTM	Posttranslational Modification
PTSD	Posttraumatic Stress Disorder
PUFA	Polyunsaturated Fatty Acids
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SAD	Social Anxiety Disorder
SAH	S Adenosyl Homocysteine
SAM	S Adenosyl Methionine
SE	Standard Error
SKA2	Spindle and Kinetochore-associated Complex Subunit 2
SLC6A2	Solute Carrier Family 6 Member 2
SLC6A3	Solute Carrier Family 6 Member 3
SLC6A4	Solute Carrier Family 6 Member 4
SLC8A2	Solute Carrier Family 8 Member 2
SNP	Single Nucleotide Polymorphism
SS	Socioeconomic Status
STAI	State-Trait Anxiety Inventory
STEAP3	Six-transmembrane Epithelial Antigen of the Prostate 3
SWAN	Subset-quantile Within Array Normalization
TA	Trait Anxiety
TNF	Tumor Necrosis Factor
TSS	Transcription Start Site
TTM	Trichotillomania
UTR	Untranslated Region
Val	Valine

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1. INTRODUCTION

1.1 ANXIETY DISORDERS

1.1.1 Introduction to Anxiety Disorders

The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), defines anxiety disorders as a wide range of complex, multifactorial disorders primarily characterised by abnormal and inappropriate anxiety in the absence of anxiety-provoking stimuli (DSM-V, American Psychiatric Association, 2013). This includes, but is not limited to, panic disorder, generalised anxiety disorder (GAD), social anxiety disorder (SAD), trichotillomania (TTM) and specific phobias such as agoraphobia. Anxiety describes four aspects of experience that all individuals, including those without a clinical disorder, may perceive: physical tension, mental apprehension, physical symptoms and dissociative anxiety (Healy, 2008). Anxiety disorders cause significant impairment (Roberts et al., 2007), including social and academic dysfunction (Langley et al., 2004), and persistent medical issues in various organ systems such as gastrointestinal disease (Shah et al., 2014), respiratory disease (Willgoss and Yohannes, 2013) and cardiovascular illness (Player and Peterson, 2011).

Anxiety disorders, along with mood disorders, are the primary cause of years lived with disability worldwide (WHO report., 2000). The lifetime occurrence of anxiety disorders differs, ranging from 2% for agoraphobia to as high as 15% for specific phobias as measured in American individuals (Kessler et al., 2005). Anxiety disorders also rank as the most prevalent class of psychiatric disorders (Kessler et al., 2005). However, prevalence also differs across gender (with higher rates in females compared to males) (McLean et al., 2011; Somers et al., 2006); age (with increasing rates after the age of 18) (Somers et al., 2006) and ethnicity (with higher rates of anxiety disorders in Caucasians compared to other ethnicities) (Asnaani et al., 2010). Furthermore, anxiety disorders often co-occur with one another and with other psychiatric disorders, including major depressive disorder (MDD) and posttraumatic stress disorder (PTSD) (Nugent et al., 2011). A South African Stress and Health study found that South Africa has relatively higher yearly prevalence of anxiety (16%) and mood disorders (10%)

compared to other countries included in the World Mental Health Survey Initiative (Herman et al., 2009).

The underlying aetiology of anxiety disorders has been attributed to complex interactive effects between genetic and environmental factors (McNaughton, 1997; Mehta and Binder, 2012; Venault and Chapouthier, 2007). Multiple twin studies have been conducted to elucidate the genetic underpinnings of the various anxiety disorders. Heritability is calculated as the proportion of observed differences within a trait among individuals of a population that are due to genetic differences (Raj and van Oudenaarden, 2008). A comprehensive heritability analysis performed by the Virginia Institute for Psychiatric and Behavioural Genetics determined GAD and panic disorder heritability to be ±30% and ±40%, respectively (Hettema et al., 2001b). In addition, family studies of probands have determined up to 16% increased susceptibility to panic disorder in first degree relatives of individuals diagnosed with panic disorder (Hettema et al., 2001a). An analysis by Kendler et al. (1999) determined heritability of specific phobias to range between 40% and 60% (Kendler et al., 1999). Multiple studies have suggested that common genetic risk factors are involved in the development of various anxiety disorders and additional co-morbid disorders (Brady et al., 2000; Fyer et al., 1996; Kendler et al., 2007; Nugent et al., 2011). Two methods of research have been primarily employed to investigate the genetic factors involved in the development of complex psychiatric disorders such as anxiety disorders. One of these; genome-wide association studies (GWAS) describes robust methods of study used to investigate diseases in a case-control manner by comparing single nucleotide polymorphisms (SNPs), variations at single nucleotides that occur at specific positions in the genome, across the entire genome of cases and controls (Hirschhorn and Daly, 2005). Thus far, very few GWAS have been conducted to investigate anxiety disorders, most likely due to limited access to sufficient sample sizes (Koenen, 2007) and inadequate matching of risk factors across the samples, especially given the phenotypic heterogeneity of anxiety disorders (Skelton et al., 2012). GWAS have been conducted to investigate panic disorder (Otowa et al., 2012, 2009; Shen et al., 2013) and specific phobias (Walter et al., 2013), but these studies remain limited due to insufficient samples sizes and lack of statistical power. However, a recent meta-analysis combining data from GWAS conducted on various anxiety disorders, has implicated SNPs in a

non-coding RNA locus as well as the calmodulin-lysine N-methyltransferase expressing gene in anxiety disorder development (Otowa et al., 2016). Candidate gene studies, whereby the associations between genetic variation in pre-selected genes and disease states are investigated, describes another method of research that has been primarily used to investigate the genetic contribution to anxiety disorders. Due to the influence that physical and psychological stress experience can exert on functioning of the hypothalamic-pituitary-adrenal (HPA) axis, as well as the influence HPA axis functioning exerts on how physical and psychological stress is experienced (Faravelli et al., 2012b), the majority of candidate gene studies have investigated genes involved in both the maintenance and the functioning of this system. The genes encoding the serotonin transporter (*SLC6A4*) (Blaya et al., 2010; Bloch et al., 2008; Hemmings et al., 2016; Narasimhan et al., 2011; Strug et al., 2010), the brain-derived neurotrophic factor (*BDNF*) (Han et al., 2015; Jiang et al., 2005; Xie et al., 2011), the glucocorticoid receptor (*NR3C1*) and its regulator (Panek et al., 2014; Xie et al., 2010), the FK506 binding protein 5 (*FKBP5*) (Laryea et al., 2013; Stephens and Wand, 2012), the catechol-O-methyltransferase (*COMT*) (Hettema et al., 2008b; Samochowiec et al., 2004), the corticotropin-releasing hormone (Smoller et al., 2005), vasopressin and CRH type 1 receptor (Keck et al., 2008) are a few of the genes that have been consistently associated with both HPA-axis functioning, as well as anxiety disorder development. However, despite concerted efforts from researchers around the globe, no single gene has been found to directly contribute to the aetiology of anxiety disorders (Gregory and Eley, 2007; Knight, 2005). One of the reasons for this could stem from the inability to detect risk genetic variants for anxiety against a backdrop of clinical heterogeneity. Several causal variants have been associated with anxiety disorders and could potentially have a collective additive effect in conferring susceptibility to develop anxiety disorders.

Childhood and adolescence represent periods in life where individuals are particularly vulnerable to stress exposure and exposure to adverse environmental factors, due to the subtle changes in functioning of the stress response that occur during these periods (Romeo, 2013). A number of these adverse environmental factors have been implicated in the influence of anxiety disorder susceptibility and development of stress-related pathology (Domschke and

Dannlowski, 2010; Heim et al., 2010; Uher, 2014; Zhang et al., 2015). They include childhood maltreatment (Cortes et al., 2005), such as physical abuse, physical neglect, sexual abuse, emotional abuse and emotional neglect, as well as negative life events (Grover et al., 2005; Tiet et al., 2001), such as loss of a family member or pet, divorce, debilitating accidents and relocating, and cognitive biases (i.e. attentional, interpretation and memory bias)(Bar-Haim et al., 2007; MacLeod et al., 1986; Muris, 2010; Waters et al., 2008). Indeed, numerous clinical and epidemiological data suggest early-life stress (ELS) is a relevant risk factor for development of both anxiety and mood disorders (Carr et al., 2013; Faravelli et al., 2012a; Kessler et al., 2010; Nugent et al., 2011). While these environmental factors increase or maintain anxiety, they do not function in isolation, rather, they interact with genetic factors to produce potentially maladaptive outcomes (Domschke and Dannlowski, 2010; Hettema et al., 2005; Nugent et al., 2011; Sharma et al., 2016).

A study previously conducted by Kessler et al. (2005) found that the onset of most anxiety disorders occurs during childhood or early adolescence, estimating the median age of onset at 11 years old (Kessler et al., 2005). Moreover, a fairly recent World Health Survey revealed that ELS is responsible for the onset of roughly 30% of anxiety disorders (Kessler et al., 2010). To this end, anxiety disorders in childhood predict a range of psychiatric disorders in adolescence (Bittner et al., 2007), including SAD, overanxious disorder, conduct disorder and depression, which subsequently predict an increased risk for recurrent psychiatric disorders in adulthood (Pine et al., 1998; Roza et al., 2003; Woodward and Ferguson, 2001). It is therefore beneficial to identify and better characterize the risk factors that result in the development of anxiety symptoms in childhood, and to identify those risk factors that mediate the persistence of anxiety symptoms into adulthood.

1.1.2 Anxiety Sensitivity and Anxiety Proneness

Anxiety Disorder Endophenotypes

Endophenotypes describe those phenotypes or measurable components along the pathway between the disease phenotype and its corresponding genotypes (Davis et al., 2002). They refer to intermediate phenotypes that are more closely associated with specific genetic variants than

the more complex phenotypic construct of the disorder (Gottesman and Gould, 2003). Many anxiety disorder endophenotypes have been characterised, including measurable elements of HPA axis dysfunction, fear responsiveness, behavioural inhibition, trait anxiety (TA) and anxiety sensitivity (AS) (D'Mello and Steckler, 1996; Domschke and Dannlowski, 2010; Landgraf and Wigger, 2003).

In the context of anxiety disorders, analysing endophenotypes has the potential to prove particularly useful when studying genetic and epigenetic associations (will be discussed at length in the following section) (Steimer, 2011). Screening for epigenetic associations could be more successful if a discrete process, likely governed by few genes, is being investigated during a study as opposed to an entire syndrome, often the product of interaction between several more genes (Davis et al., 2002). In relation to anxiety disorders, not all affected individuals present with similar clinical phenotypes and as a result, genetic and epigenetic associations that could be observed are likely to be varied. Therefore, the heterogeneity that accompanies anxiety disorder diagnosis has the potential to reduce the statistical power of a given study (Davis et al., 2002).

Anxiety Sensitivity and Anxiety Proneness

Anxiety sensitivity (AS) is a well-established anxiety disorder endophenotype (Bennet and Stirling, 1998; Grant et al., 2007; Naragon-Gainey, 2010; Plehn and Peterson, 2002). It describes the dispositional fear of anxiety-related sensations or bodily symptoms, based on the belief that these symptoms could be physically or psychologically harmful (Reiss, 1991). Twin and family studies have reported a genetic basis for AS, and heritability is estimated to range between 45% and 50% (Eley et al., 2003; Stein et al., 1999; Zavos et al., 2012a). Indeed, multiple genes have been associated with AS, including *SLC6A4* (Hemmings et al., 2016; Stein et al., 2007a; Zavos et al., 2012b), *COMT* (Baumann et al., 2013) and the neuropeptide S receptor 1 gene (*NPSR1*) (Klauke et al., 2014).

Trait anxiety (TA), defined as the general tendency to respond anxiously or fearfully to anxiety-provoking stressors (McNally, 1989; Reiss, 1997), is another well-established anxiety disorder

endophenotype that has been implicated in the development of anxiety and depression (Grupe and Nitschke, 2013).

In the current study, anxiety proneness (AP) is defined as a measure of the two aforementioned anxiety-related temperamental traits. Therefore, individuals with inflated levels of AS and TA are classified as anxiety prone, and are at increased risk of developing an anxiety disorder (Simmons et al., 2006). However, AP has been defined and conceptualised in several different ways, including as a measure of TA exclusively (Spielberger, 2013), a measure of behavioural inhibition (Gray, 1987), a measure neuroticism and extraversion (Stein et al., 2005) and a measure of harm avoidance (Cloninger et al., 1998). In all of the aforementioned studies, AP prospectively predicts development of anxiety disorders, and acts as a potential cognitive endophenotype for these disorders (Schmidt et al., 1997). A monozygotic and dizygotic twin study has established a genetic basis for AP, defined as a compound of the five aforementioned AP constructs (Gustavsson et al., 1996), however, to date, no candidate gene studies in the context of AP have been conducted. Neural deficits have been associated with increased AP, with neuroimaging studies suggesting the involvement of increased amygdala- and insula reactivity in development and maintenance of anxiety proneness (Bishop, 2007; Paulus et al., 2004; Paulus and Stein, 2006; Simmons et al., 2006; Stein et al., 2007b).

As is the case with anxiety disorders, environmental factors, in particular, psychosocial factors, play a crucial role in the development of both AS and anxiety proneness. Among others, negative family environments, academic difficulties, loss or separation and social adversity have consistently been associated with the development of AS (Grover et al., 2005; Scher and Stein, 2003; Stein et al., 1999). Since the interaction between genetic and environmental factors play an important role in the development of anxiety disorders and its endophenotypes, it is imperative to study this interaction. One mechanism of action whereby the environment and genes may interact is via epigenetics.

1.2 THE ROLE OF DNA METHYLATION IN ANXIETY DISORDERS

1.2.1 Introduction to Epigenetics

Epigenetics is defined as the study of mitotically and meiotically heritable changes in gene function that are not attributable to changes in the DNA sequence (Russo et al., 1996). This form of heritable transcriptional control provides an additional mechanism for regulating gene expression (Bjornsson et al., 2004).

Epigenetic gene regulation can be categorised into four groups: (i) histone post-translational modifications (PTMs), which include acetylation, methylation, phosphorylation, ubiquitination and sumoylation, and histone variant exchange; (ii) chromatin remodelling; (iii) DNA methylation and (iv) non-coding RNA-mediated modifications (Bagot et al., 2014). Generally, epigenetic modifications are very stable, but they do have the potential to be reversed, providing a potential mechanism to mediate the interaction between genetic predispositions, changes in neural function, behaviour and environmental factors (Jaenisch and Bird, 2003). It has previously been shown that epigenetic marks have the potential to be altered under different environmental influences, notably diet, pharmaceuticals and maternal care (Abel and Poplawski, 2014). In addition, previous studies have observed stable epigenetic marks that contribute to an anxiety-like phenotype in adult rats as a result of ELS (Blaze et al., 2013). Several animal studies have previously associated epigenetic modifications with learning and memory processes, activity-dependent neuronal gene expression and disease states such as depression, stress and addiction (Anier et al., 2010; Chen et al., 2003; Chwang et al., 2006; Kumar et al., 2005; Martinowich et al., 2003; Morris and Monteggia, 2014; Schwartzentruber et al., 2012; Weaver et al., 2004).

As the mechanisms of action, as well as the methods for investigation of DNA methylation is well understood, this epigenetic mechanism was selected as the best candidate to investigate the study question. Therefore, for the purpose of this study, the rest of the introduction will primarily focus on DNA methylation.

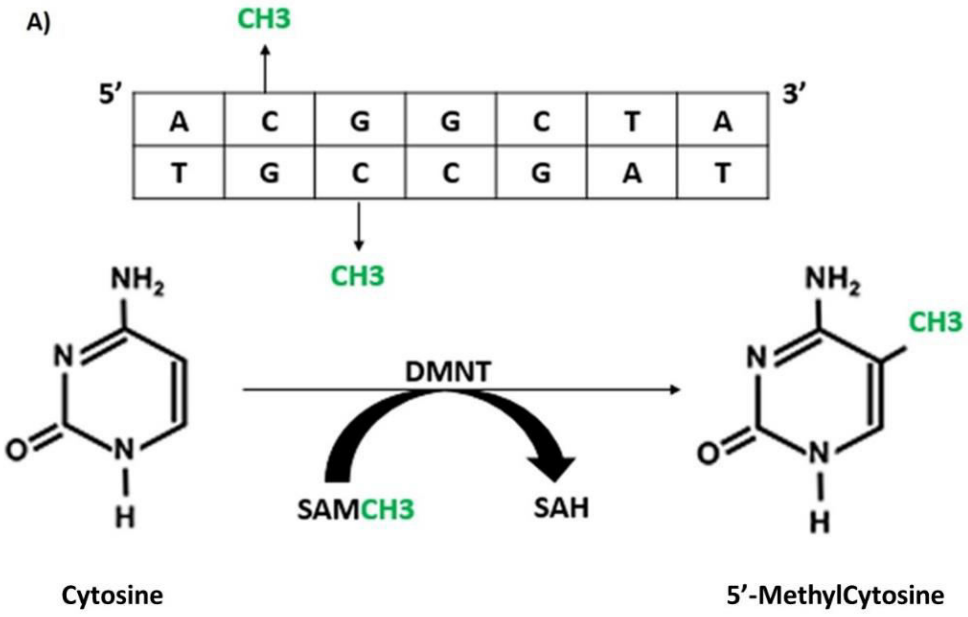
1.2.2 DNA Methylation

Of the aforementioned epigenetic modifications, DNA methylation is the most well-studied and investigated (Novik et al., 2002). DNA methylation has been implicated in multiple developmental processes such as X-chromosome inactivation, genomic imprinting, and maintaining genomic stability (Novik et al., 2002). DNA methylation has also been linked to tissue – specific and –appropriate gene expression patterns during mitosis, as well as repression of retrotransposons and foreign elements (Klose and Bird, 2006; Maunakea et al., 2010; Sasaki and Matsui, 2008).

In mammals, the mechanism of DNA methylation involves the binding of a methyl group to the C5 position of the nucleobase cytosine, thus forming 5-methylcytosine (5mC), positioned in CpG dinucleotides (Klose and Bird, 2006; Reik and Dean, 2001) as illustrated in Figure 1 (A). Universally, ±75% of CpG dinucleotide sites within the human genome are methylated, although this varies during early development (Ehrlich et al., 1982).

The DNA methylation process requires essential enzymes to catalyse the transfer of the methyl group to the cytosine, called DNA methyltransferases (DMNTs). Three active DMNTs are involved in this action: DMNT1, DNMT3A and DNMT3B. DNMT1 is responsible for the maintenance of DNA methylation patterns between parent and daughter cells during cell development (Morris and Monteggia, 2014; Turek-Plewa and Jagodziński, 2005). It does so by binding methyl groups to hemi-methylated CpG regions. DNMT3A and DNMT3B catalyse *de novo* DNA methylation on both hemi-methylated and unmethylated CpG regions (Turek-Plewa and Jagodziński, 2005; Xie et al., 1999). Additionally, another two DMNTs have been identified: DNMT3L, an accessory protein which is catalytically inactive but stimulates the catalytic activity of DMNT3A and DNMT3B in embryonic stem cells, and DNMT2, which has been implicated as an RNA methyltransferase (Goll et al., 2006; Jurkowski et al., 2008; Kiani et al., 2013; Turek-Plewa and Jagodziński, 2005). Previous reports have noted the abundance of DNMT1 and DNMT3A in the mature brain, whilst DNMT3B and DNMT3L were undetectable (Feng et al., 2010). DNMT1, 3A and 3B are essential for normal prenatal development as DNMT knockouts result in lethal consequences with regard to mammalian embryos (Xie et al., 1999).

As a result of DNA methylation, methyl CpG-binding domain proteins are recruited to the methylated regions. In turn, these proteins interact with histone-modifying enzymes, chromatin remodelling complexes and DNMTs to activate, in most cases, gene silencing by modifying chromatins in a way that makes it inaccessible for transcription-inducing proteins (Murgatroyd et al., 2009).



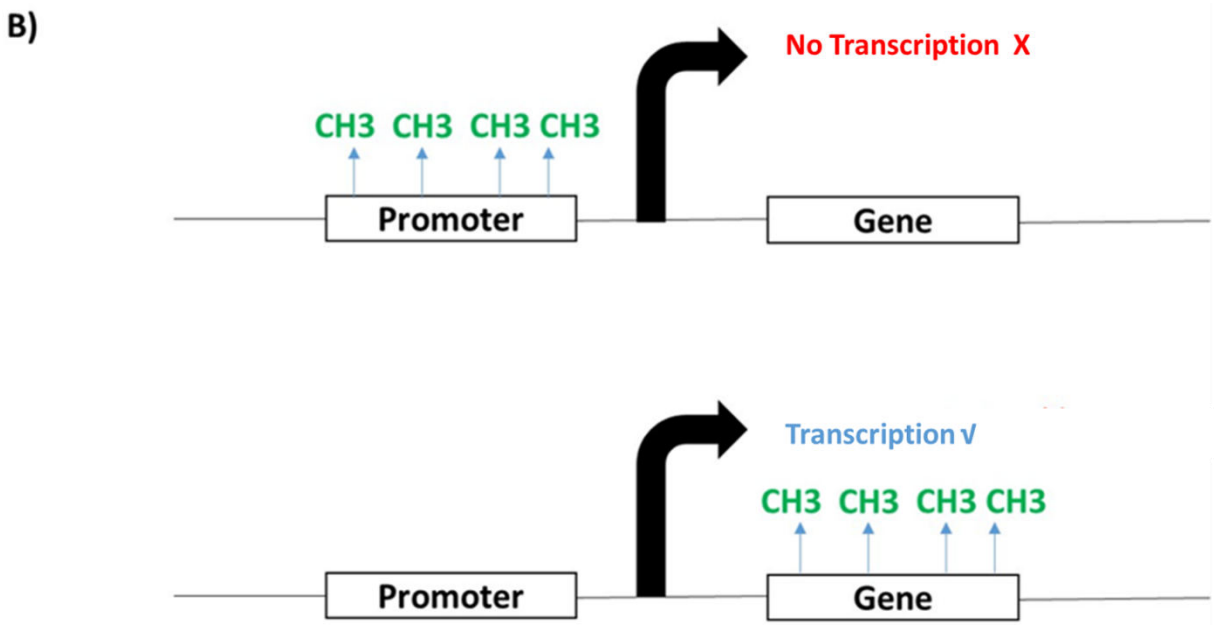


Figure 1: A) The mechanism by which cytosines in CpG sites are converted to 5-methylcytosines. B) The mechanism by which DNA methylation influences gene expression. CH3 – methyl group; DMNT – DNA methyltransferase; SAM – S adenosyl methionine; SAH – S adenosyl homocysteine.

However, the effect DNA methylation has on gene expression is dependent upon where in the gene the DNA methylation takes place (Jones, 1999). Methylation within close range to the transcription start site (TSS) inhibits transcription factors and RNA polymerase access to the DNA, and results in gene silencing (Strathdee and Brown, 2002; Turner, 2002). Protein complexes that support histone de-acetylation and gene repression are subsequently recruited (Jones et al., 1998). In contrast, DNA methylation within the gene body does not necessarily result in gene silencing and could potentially prompt continuous transcription (Moarefi and Chédin, 2011). The mechanism by which DNA methylation may influence gene expression is illustrated by Figure 1 (B). Indeed, gene body methylation is characteristic of transcribed genes and it has also been linked to gene splicing (Wolf et al., 1984). A limited number of CpG dinucleotides are found within the majority of gene bodies, instead they contain numerous repetitive and transposable elements (Jones, 2012). Thus, the presence of 5mC in the gene body or promoter region is not enough to elicit a transcriptional response. Interpretation of 5mC in genomic context is vital. Since most genes have at least two TSSs, methylation could support regulation of alternative promoter usage (Jones, 2012).

Mammalian cells have the ability to reconfigure DNA methylation patterns in response to stimuli in a fast and flexible manner (Klengel et al., 2013). Passive de-methylation occurs when maintenance methylation does not occur during cell division due to defective *DMNT1* expression. However, direct removal of a methyl group is unlikely as it requires a significant amount of energy. Thus the mechanism by which de-methylation/methylation reconfiguration in response to stimuli takes place in a fast and efficient manner is not yet known (Klengel et al., 2014). Nonetheless, although controversial, several mechanisms of active de-methylation have been proposed (Klengel et al., 2014), including active de-methylation through proteins that remove methylcytosines by DNA repair mechanisms, through proteins that interact with

methylcytosines by DNA modifications and by proteins that indirectly interact with methylcytosines (Klengel et al., 2014). DNA hydroxymethylation is the process of oxidisation of 5mC to form 5-hydroxymethylcytosine (Penn et al., 1972). Although the function of 5-hydroximethylcytosine formation is yet to be determined, it has been suggested that it is essential for de-methylation, whereby it prohibits DMNTs to effectively maintain methylation patterns (Guo et al., 2011; Tahiliani et al., 2009). The influence of hydroxymethylation is an important factor to take into account when conducting DNA methylation studies and interpreting the results to these studies, as the large majority of methods currently used to investigate DNA methylation do not account for hydroxymethylated sites and region.

Animal and cell culture studies have previously suggested that alterations in DNA methylation patterns may be reversible, however these findings have yet to be extrapolated to humans (Baysan et al., 2014; Herb et al., 2012; Tajerian et al., 2013; Weaver et al., 2004). Similarly, multiple studies have displayed the potential of intergenerational transmission of DNA methylation patterns in animals and by proteins, however evidence of this phenomenon in humans is still lacking and highly debated (Feil, 2009; Platonov and Isaev, 2006; Reik and Walter, 2001). However, a number of these studies have provided evidence for intergenerational impact of external environmental factors such as intimate partner violence and parental exposure to extreme traumatic conditions, on DNA methylation patterns (Radtke et al., 2011; Yehuda et al., 2015a). Therefore, when conducting DNA methylation studies, the influence of intergenerational transmission cannot be excluded as a possible confounder in the outcome.

Impaired regulation of DNA methylation can result in defective transcriptional control and lead to dysregulated gene expression, therefore increasing the risk for the development of maladaptive psychiatric outcomes (Novik et al., 2002). Although the mechanisms of several DNA methylation-related processes are not yet fully understood; the marked influence environmental exposure, and early life stress in particular, have on DNA methylation patterns during an individual's formative years has been well-studied (Novik et al., 2002).

1.2.3 Early Life Stress and DNA Methylation

Differences in early life experience is one of the most common reasons for differences in health and disease progression (Heim et al., 2010). As previously mentioned, dysregulation of HPA-axis functioning is a common feature in several psychiatric disorders, including anxiety disorders (Arborelius et al., 1999; Heim et al., 2010). As a result, the large majority of epigenetic research investigating anxiety disorders and early life stress has focused on genes encoding components involved in HPA-axis functioning.

Although the epigenome is under considerable environmental influence well into adulthood, early postnatal life and adolescence, in particular, represent periods of life which are regarded as environmentally-sensitive periods of life during which the environment is essential in shaping an individual's epigenome (Roth and Sweatt, 2011). These early, stable epigenomic changes have the potential to alter central nervous system (CNS) and HPA axis functioning, and thus increase (or decrease) the risk for developing adult psychopathology (Roth and Sweatt, 2011). Multiple rodent studies have provided evidence supporting the hypothesis that epigenetic modifications underlie the long-term changes that are brought about by early life environment and stress (Simmons et al., 2012; Weaver et al., 2004).

A hallmark study in this regard was conducted by Weaver et al. (2004). Here, the influence of maternal behaviour (licking and grooming) on epigenetic modifications in the male offspring was investigated. Adult offspring of mothers exhibiting high levels of licking and grooming were found to be more resistant to developing anxiety-like behaviours at a later stage in life, compared to offspring who received lower levels of licking and grooming from their mothers. This effect was attributed to DNA methylation in the promoter region of *NR3C1* in the rat hippocampus. Both the glucocorticoid receptor, as well as the hippocampus, have previously been implicated as important regulators of the stress response system (Laryea et al., 2013; Phillips et al., 2006). These differences were observed as early as the first week of life and lasted into adulthood. Furthermore, the effect was found to be reversed by means of cross-fostering (Weaver et al., 2004).

A study conducted by McGowan et al. (2009) translated the findings of made by Weaver et al. (2004) to humans by focusing their research on the effect that ELS, in the form of childhood trauma, may have on the DNA methylation pattern of *NR3C1* in the hippocampus of male suicide victims. The study was conducted in suicide victims (with history of childhood trauma), suicide victims (without history of childhood trauma) and controls (individuals who died of natural causes). Significantly increased *NR3C1* methylation was observed in male suicide victims with a history of childhood abuse, compared to the other two groups. No significant difference in the methylation status of the *NR3C1* was found in suicide victims without a history of childhood abuse and controls (McGowan et al., 2009). Similar epigenetic modifications within *NR3C1* have been associated with childhood trauma in MDD patients (Alt et al., 2010) and positively correlated with childhood trauma severity (Perroud et al., 2011). Moreover, differential DNA methylation within exons of *NR3C1* has also been associated with childhood abuse in suicide completers, as measured in hippocampal tissue (Labonte et al., 2012b) and significantly elevated methylation of the *NR3C1* promoter as a result of childhood maltreatment has also been observed when measured in leukocytes (Tyrka et al., 2012).

History of childhood abuse has been found to significantly influence the methylation pattern of the oxytocin receptor gene (*OXTR*), in a large sample of African Americans (Smearman et al., 2016), as well as the ribosomal RNA gene in a sample of Caucasian suicide completers (McGowan et al., 2008). Similarly, the effect of parenting, as measured between the ages of 11 and 13, on young adult health has been found to be mediated by methylation within the tumor necrosis factor gene (*TNF*) (Beach et al., 2015).

Numerous epigenome-wide association studies (EWAS) have been conducted to investigate the influence of ELS and childhood trauma on methylation patterns. Differential methylation, as measured during adulthood, of several loci has been associated with severe childhood abuse (Labonté et al., 2012a; Mehta et al., 2013; Suderman et al., 2014) as well as childhood socioeconomic status (Borghol et al., 2011; Swift-Scanlan et al., 2014; Uddin et al., 2013). Learning and memory genes, in particular, were found to be significantly differentially methylated in suicide victims, with a history of sexual or physical abuse, when compared to

healthy controls, illustrating the impact of child abuse (Labonté et al., 2012a), while methylation within stress- and inflammation-related genes were found to be significantly increased when comparing between low- and high childhood socioeconomic status (Needham et al., 2015).

These studies are indicative of the marked influence early life experience has on both DNA methylation signatures as well as risk to develop psychopathology during adolescence and adulthood, and are summarised in Table 1. Additionally, these studies illustrate the importance of taking early life experience into account when conducting epigenetic investigations into anxiety disorders.

Table 1: DNA methylation studies investigating early-life stress and childhood trauma				
Population/Ethnicity (cases: controls)	Sample/Source	Investigated Region	Findings	Reference
Childhood Trauma				
Mixed American** (n=85*)	Whole blood	EWAS	Methylation in the Kit ligand gene mediates the relationship between childhood trauma and cortisol stress reactivity.	(Houtepen et al., 2016)
Mixed American (61:108)	Whole blood	EWAS	Differential DNA methylation in individuals with and without childhood trauma.	(Mehta et al., 2013)
French-Canadian (25:16)	Whole blood	EWAS	362 differentially methylated promoters in individuals with a history of abuse vs. controls.	(Suderman et al., 2014)
Mixed American (n= 76*)	Whole blood	*FKBP5*	Observed de-methylation due to childhood trauma.	(Klengel et al., 2013)
Caucasian (24:12)	Brain tissue (hippocampal)	*NR3C1*	Greater *NR3C1* promoter methylation in suicide victims with childhood trauma vs. those without.	(McGowan et al., 2009)
Caucasian (n = 112*)	Brain tissue (hippocampal)	*NR3C1*	History of childhood abuse associated with site-specific *NR3C1* promoter methylation.	(Labonte et al., 2012b)
Caucasian (n = 215*)	Leukocytes	*NR3C1*	Childhood sexual abuse and its severity is significantly positively correlated with *NR3C1* methylation.	(Perroud et al., 2011)
Mixed American (n= 99*)	Leukocytes	*NR3C1*	Childhood maltreatment associated with elevated *NR3C1* promoter methylation.	(Tyrka et al., 2012)

African American (n-393*)	Whole blood	*OXTR*	Childhood abuse interacted with CpG methylation to predict psychopathology.	(Smearman et al., 2016)
Caucasian (13:11)	Brain tissue (hippocampal)	ribosomal RNA gene	History of childhood abuse associated with hypermethylation.	(McGowan et al., 2008)
Socioeconomic Status				
Mixed American (23:77)	Whole blood	EWAS	Socioeconomic status modulates relationship between methylation and risk to stress-related pathology.	(Uddin et al., 2013)
British (n= 40*)	Whole blood	EWAS	Methylation levels of 1252 gene promoter regions were associated with childhood socioeconomic status.	(Borghol et al., 2011)
Mixed American (n = 1264*)	Monocytes	18 stress-reactivity- and inflammation-related genes	Socioeconomic status associated with differential methylation of several genes, including *FKBP5* and *OXTR.*	(Needham et al., 2015)
Mixed American (n= 48)	Saliva	*COMT*	Socioeconomic status associated with methylation of multiple *COMT* CpG sites.	(Swift-Scanlan et al., 2014)
Other Early Developmental Factors				
African American (n= 398)	Monocytes	*TNF*	*TNF* methylation acts as significant mediator of the association of parenting with young adult health.	(Beach et al., 2015):

Abbreviations: CpG – cytosine-guanine dinucleotide site; EWAS – epigenome-wide association study; FKBP5 – FK506 Binding Protein 5; NR3C1 – nuclear receptor subfamily 3 group C member 1; OXTR – oxytocin receptor; COMT - catechol O-

methyltransferase; TNF – tumor necrosis factor; RNA – ribonucleic acid.

Footnotes:

*** Sample cells that indicate n refer to the number of individuals included in the study (not divided into cases and controls), either all were exposed to varying degrees of childhood trauma/socioeconomic background or categorised by disease state in the related study.**

**** Mixed American refers to American individuals of mixed ancestry including African, Asian, Caucasian and Hispanic ancestry.**

1.2.4 DNA Methylation and Anxiety Disorders

The significant influence prenatal-, early life- and chronic stress exercise over DNA methylation patterns throughout the genome has been displayed consistently. Consequently, a considerable amount of research has attempted to identify whether these same changes in DNA methylation patterns, brought about by the aforementioned environmental factors, are found in disease states such as anxiety disorders and its comorbid disorders e.g. PTSD and MDD.

Three candidate gene epigenetic studies have been conducted, investigating panic disorder (Bayles et al., 2013; Domschke et al., 2013, 2012). Hypomethylation of regions within both the glutamate decarboxylase 1 gene (*GAD1*) and the monoamine-oxidase A gene (*MAOA*), as measured in whole blood, have been observed. Significantly reduced *MAOA* methylation (promoter and exon/intron 1) has been associated with traumatic life events as well as adjustment disorder events in female panic disorder patients compared to healthy female controls, exclusively, while positive life events were associated with increased methylation of the same *MAOA* region (Domschke et al., 2012). Negative life events, along with the *GG* genotype of the *GAD1* SNP, rs3762555, were found to mediate reduced methylation in the promoter and intron 2 region of *GAD1* in panic disorder patients compared to healthy controls. This effect was also found to be female-specific (Domschke et al., 2013). Hypomethylation has also been observed in the solute carrier family 6 member 2 gene (*SLC6A2*), as measured in whole blood, however no significant differences were found between panic disorder patients and controls (Bayles et al., 2013).

Significantly elevated levels of global DNA methylation, as measured in whole blood, have been observed in anxious individuals compared to non-anxious individuals (Murphy et al., 2015). This universal hypermethylation was found to influence the expression of *DNMT1*, *DNMT3A* and *DNMT3B*, which in turn influenced the severity of anxiety as assessed by the anxiety questionnaire, HADS-A (Murphy et al., 2015). A similar significant increase in *SLC6A4* methylation, as measured in DNA extracted from saliva, within the gene's promoter region has previously been observed in patients diagnosed with a DSM-V categorised anxiety disorder, who responded successfully to cognitive behavioural therapy (CBT) compared to patients who

did not respond to CBT (Roberts et al., 2014). Conversely, a significant reduction in methylation in the third exon of OXTR, as measured in whole blood, has been associated with increased risk to develop SAD (Ziegler et al., 2015). This reduction *OXTR* methylation has been hypothesised to act as either a causal activator of social anxiety or as a possible compensatory mechanism in response to reduced systemic oxytocin (Ziegler et al., 2015). Furthermore, two candidate gene epigenetic studies have investigated DNA methylation patterns in combined samples of patients with anxiety disorders and depression (Chagnon et al., 2015; van der Knaap et al., 2015). While one study found a trend towards a significantly positive association between *NR3C1* methylation, as measured in whole blood, and risk to develop either disorder (van der Knaap et al., 2015), the other study found significantly greater methylation in both *BDNF* as well as *OXTR* (as a function of the rs53576 genotype), as measured in saliva, associated with anxiety/depression (Chagnon et al., 2015).

A number of EWAS have been conducted in PTSD cohorts, yielding interesting results (Mehta et al., 2013; Smith et al., 2011; Uddin et al., 2010). Two of these studies have identified genes involved in the immune system as differentially methylated in PTSD patients compared to controls, as measured in whole blood (Smith et al., 2011; Uddin et al., 2010). Several candidate gene methylation studies investigating PTSD have also yielded notable results (Bam et al., 2016; Ressler et al., 2011; Rusiecki et al., 2013, 2012; Uddin et al., 2011a). Differential methylation in the spindle and kinetochore-associated complex subunit 2 gene (*SKA2*) has been associated with emergence of PTSD symptoms (Boks et al., 2015), susceptibility to PTSD (Kaminsky et al., 2015) and PTSD severity (Sadeh et al., 2016). Similarly, differential methylation, as measured in whole blood, in *COMT* (Norrholm et al., 2013) has been associated with impaired fear inhibition in PTSD while differential methylation in *NR3C1*, as measured in whole blood and saliva (Vukojevic et al., 2014; Yehuda et al., 2015b), *DNMT1*, as measured in whole blood and saliva (Sipahi et al., 2014), *FKBP5*, as measured in whole blood and saliva (Klengel et al., 2013), the solute carrier family 6 member 3 gene (*SLC6A3*), as measured in whole blood and saliva (Chang et al., 2012) and *SLC6A4*, as measured in whole blood and saliva (Koenen et al., 2011) have all been associated with regard to increased risk to develop PTSD.

Although MDD is not categorised as an anxiety disorder, with regards to genetics research, the influence of childhood environment, demographics and personality traits, MDD and anxiety disorders share significant overlap (Hettema, 2008b). As is the case in PTSD, multiple EWAS have been conducted in MDD research (Byrne et al., 2013; Córdova-Palomera et al., 2015; Sabunciyan et al., 2012; Uddin et al., 2011b). Notably, two studies conducted in monozygotic twins have displayed the influence of both differential methylation and variable methylation (between twins) in depression (Byrne et al., 2013; Córdova-Palomera et al., 2015). Additionally, multiple differentially methylated genes have been identified via candidate gene epigenetic studies (Haghighi et al., 2015; Zill et al., 2012) while many of the genes found to be differentially methylated in PTSD and panic disorder have also been implicated in targeted MDD methylation analysis, including *BDNF*, as measured in whole blood (Carlberg et al., 2014; Dell'Osso et al., 2014; Fuchikami et al., 2011; Kang et al., 2013a; Kim et al., 2015), *SLC6A4*, as measured in whole blood (Alasaari et al., 2012; Kang et al., 2013b; Zhao et al., 2013), *FKBP5*, as measured in whole blood (Höhne et al., 2015) and *MAOA*, as measured in saliva (Melas et al., 2013). Table 2 summarises the DNA methylation studies that have been conducted in humans with regards to anxiety- and stress-related phenotypes, including anxiety disorders, PTSD and MDD. In addition, alterations in DNA methylation patterns, as measured in various tissue, of several of the genes implicated in HPA-axis functioning have been associated with other psychiatric disorders, including *BDNF* and *COMT* in schizophrenia (Abdolmaleky et al., 2006; Ikegame et al., 2013) and bipolar disorders (Dell'Osso et al., 2014; Strauss et al., 2013), *NR3C1* in externalising disorders (Heinrich et al., 2015), *COMT* in substance abuse disorders (van der Knaap et al., 2014) and *BDNF* in borderline personality disorder (Thaler et al., 2014).

The aforementioned studies highlight the large extent to which DNA methylation has been investigated in psychiatric illnesses, however it also highlights the lack of DNA methylation studies that have investigated anxiety disorders specifically. Despite the importance of AS and AP as anxiety disorder risk factors and endophenotypes, DNA methylation studies investigating AS and AP are yet to be conducted. Therefore it is crucial to conduct a DNA methylation study investigating AS and AP, in order to broaden our understanding of the endophenotype, as well

as investigating the influence DNA methylation may play in the persistence of AS and AP into adulthood and development into anxiety disorders.

Table 2: DNA methylation studies investigating stress- and anxiety-related psychopathology.				
Population/Ethnicity (cases: controls)	Sample/Source	Investigated Region	Findings	Reference
Anxiety Disorders				
English (25:22)	Whole blood	EWAS	Significantly increased global DNA methylation in anxious vs. non-anxious individuals	(Murphy et al., 2015)
Mixed American (n= 116*)	Saliva	*SLC6A4*	Differential *SLC6A4* methylation in treatment responders vs. non-responders	(Roberts et al., 2014)
French-Canadian (19:24)	Saliva	*SLC6A4, BDNF, OXTR*	Significantly elevated methylation in *BDNF* and *OXTR* (in *AA* genotype of rs53576)	(Chagnon et al., 2015)
Dutch (n= 412*)	Whole blood	*SLC6A4* and *NR3C1*	*NR3C1* methylation associated with lifetime risk to internalising disorder	(van der Knaap et al., 2015)
Social Anxiety Disorder				
Caucasian (111:111)	Whole blood	*OXTR*	Significantly decreased *OXTR* methylation was associated with SAD	(Ziegler et al., 2015)
Panic Disorder				
German (65:65)	Whole blood	*MAOA*	Panic disorder associated with *MAOA* hypomethylation	(Domschke et al., 2012)
German (65:65)	Whole blood	*GAD1*	Panic disorder associated with *GAD1* hypomethylation	(Domschke et al., 2013)
PTSD				

Mixed American*** (61:108)	Whole blood	EWAS	Differential gene expression due to methylation in PTSD patients with and without childhood trauma	(Mehta et al., 2013)
African American (50:60)	Whole blood	EWAS	Differential global methylation in 5 genes found in cases vs. controls	(Smith et al., 2011)
Mixed American (23:77)	Whole blood	EWAS	Socioeconomic status modulates relationship between methylation and risk to PTSD	(Uddin et al., 2013)
Mixed American (23:77)	Whole blood	EWAS	Immune system genes overrepresented as uniquely methylated	(Uddin et al., 2010)
Rwandan (93:59)	Saliva	*NR3C1*	Differential *NR3C1* methylation associated with impaired memory-related functions in PTSD.	(Vukojevic et al., 2014)
Mixed American (61:61)	Whole blood	*NR3C1*	Significantly reduced *NR3C1* promoter methylation in combat veterans with PTSD vs. without	(Yehuda et al., 2015)
Mixed American (n=76*)	Whole blood	*FKBP5*	Risk to stress-related psychiatric disorders due to allele-specific, childhood trauma dependant de-methylation	(Klengel et al., 2013)
Mixed American (n= 100*)	Whole blood	*SLC6A4*	*SLC6A4* promoter methylation modifies risk effect of traumatic exposure on PTSD	(Koenen et al., 2011)
Mixed American (16:67)	Whole blood	*SLC6A3*	High *SLC6A3* methylation combined with 9R allele confers risk to PTSD	(Chang et al., 2012)
Mixed American (98:172)	Whole blood	*COMT*	*COMT* promoter hypermethylation associated with hampered fear inhibition	(Norrholm et al., 2013)

Mixed American (30:30)	Whole blood	*DNMT1, 3A, 3B, 3L*	Increased *DNMT1* methylation following trauma exposure in PTSD patients vs. controls	(Sipahi et al., 2014)
Mixed American (16:17)	PBMC	*IL-12B*	Differential *IL-12B* methylation in PTSD vs. controls	(Bam et al., 2016)
Mixed American (n= 64*)	Whole blood	*ADCYAP1* and *ADCYAP1R1*	PTSD associated with *ADCYAP1R1* methylation	(Ressler et al., 2011)
Mixed American (75:75)	Whole blood	*LINE1*	Hypomethylation in cases vs. controls	(Rusiecki et al., 2012)
Mixed American (74:74)	Whole blood	*IGF2, IL8, IL16, IL18*	*H19* and *IL18* hypomethylation in controls vs. *IL18* hypermethylaton in cases	(Rusiecki et al., 2013)
Mixed American (23:77)	Whole blood	33 candidate genes	Increased *MAN2C1* methylation and higher traumatic event exposure confers risk to PTSD	(Uddin et al., 2011a)
Major Depressive Disorder/Depression				
Northern European (24:24**)	Whole blood	EWAS	Increased variable methylation in twin cases vs. controls.	(Byrne et al., 2013)
Caucasian Spanish (n= 34**)	Whole blood	EWAS	Multiple loci variably methylated among twins	(Córdova-Palomera et al., 2015)
Mixed American (39:26)	Brain tissue (frontal cortical)	EWAS	Multiple differentially methylated loci in cases vs. controls	(Sabunciyan et al., 2012)

Mixed American (33:67)	Whole blood	EWAS	Differential methylation in lifetime depression cases vs. controls	(Uddin et al., 2011)
Dutch (6:6)	Brain tissue (amygdalae and hippocampal)	*NR3C1*	Differential expression in MDD not due to DNA methylation differences	(Alt et al., 2010)
Swedish (392:1276)	Saliva	*NR3C1* and *MAOA*	Decreased *MAOA* methylation in cases vs. controls	(Melas et al., 2013)
Finnish (n= 49*)	Whole blood	*SLC6A4*	Highly stressed individuals had significantly lower promoter methylation compared to low stressed nurses	(Alasaari et al., 2012)
Korean (n= 108*)	Whole blood	*SLC6A4*	Increased promoter methylation associated with clinical presentation	(Kang et al., 2013b)
Caucasian (84:84)	Whole blood	*SLC6A4*	Variable promoter methylation associated with variance in depressive symptoms	(Zhao et al., 2013)
Caucasian (n= 105*)	Whole blood	*SLC6A4*	Elevated *SLC6A4* methylation as a function of recent depressive symptoms	(Duman and Canli, 2015)
German (n= 94*)	Whole blood	*SLC6A4*	Lower *SLC6A4* methylation associated with impaired antidepressant treatment response.	(Domschke et al., 2014)
Korean (n= 108*)	Whole blood	*BDNF*	Increased promoter methylation associated with poor treatment response and suicide attempts.	(Kang et al., 2013a)

Japanese (20:18)	Whole blood	*BDNF*	Significant differential *BDNF* methylation in cases vs. controls	(Fuchikami et al., 2011)
Caucasian (61:55)	Whole blood	*FKBP5*	Elevated intron 7 genotype-dependant methylation in MDD cases vs. controls	(Höhne et al., 2015)
Mixed American (61:59)	Whole blood	PUFA-related genes	Methylation significantly associated with MDD diagnosis	(Haghighi et al., 2015)
Caucasian (81:81)	Whole blood	*ACE*	*ACE* promoter hypermethylation associated with MDD	(Zill et al., 2012)

Abbreviations: PTSD – posttraumatic stress disorder; MDD – major depressive disorder; SAD – Social Anxiety Disorder; CpG – cytosine-guanine dinucleotide site; EWAS – epigenome-wide association study; DNA – deoxyribonucleic acid; PBMC – peripheral blood mononuclear cells; SLC6A3 – solute carrier family 6 member 3; SLC6A4 – solute carrier family 6 member 4; FKBP5 – FK506 Binding Protein 5; NR3C1 – nuclear receptor subfamily 3 group C member 1; OXTR – oxytocin receptor; COMT – catechol O-methyltransferase; MAOA – monoamine oxidase A; GAD1 – glutamate decarboxylase 1; DNMT – DNA methyltransferase; IL – interleukin; ADCYAP1 – adenylate cyclase activating polypeptide 1; ADCYAP1R1 – ADCYAP1 receptor type I; LINE1 – long interspersed nuclear element 1; IGF – insulin-like growth factor; MAN2C1 – mannosidase alpha class 2C member 1; PUFA – polyunsaturated fatty acids; ACE – angiotensin-converting enzyme.

Footnotes:

*** Sample cells that indicate n refer to the number of individuals included in the study (not divided into cases and controls).**

****Study conducted in twins, thus pairs of twins are used in cases and controls.**

***** Mixed American refers to American individuals of mixed ancestry including African, Asian, Caucasian and Hispanic**

ancestry.

1.3 AIMS AND OBJECTIVES

1.3.1 Aim

The overall aim of the present study is to investigate the role that genome-wide alterations in DNA methylation play in the development of AP in South African adolescents by analysing and comparing the DNA methylation profiles of i) individuals with high levels of anxiety proneness, and who have experienced high levels of childhood trauma; ii) individuals who have high levels of anxiety proneness but have not experienced high levels of childhood trauma; iii) individuals who have low levels of anxiety proneness but have experienced high levels of childhood trauma and iv) individuals who have low levels of anxiety proneness and who have not experienced high levels of childhood trauma.

The current study is nested in a larger study, the aim of which is to investigate the aetiology of AP and compare neurocognitive deficits and brain functional activity among healthy adolescents with high and low levels of childhood trauma and high and low levels of AP (Martin et al., 2014). Investigating the aetiology of AP in adolescents with early developmental trauma provides a unique opportunity to examine the interactions between childhood trauma and genetic and epigenetic factors, and to investigate how these interactions mediate the development of neural deficits associated with increased levels of AP, which in turn, is highly predictive of anxiety disorders. Therefore, the study has the potential to yield new insights into the molecular neurobiology of anxiety disorders, and could have direct implications for future studies focusing on anxiety disorders in adolescents.

1.3.2 Objective(s)

i) Conduct genome-wide methylation analysis using the Illumina Infinium 450K BeadChip array in a sample of 46 South African adolescents.

ii) Verify selected findings using the EpiTYPER MassARRAY technology by Sequenom within a sample of 63 South African adolescents, including the 46 adolescents investigated using the BeadChip array.

iii) Conduct the appropriate statistical analysis in order to identify statistical trends across the four groups of interest consisting of 63 South African adolescents.

2. METHODS

2.1 Sample & Procedure

Participants were recruited from a representative sample of secondary schools in the city of Cape Town, South Africa and its surrounds and were screened by a trained research psychologist. All participants were adolescents aged 13 to 18 years (mean age = 16.24) who have been exposed to relatively high levels of childhood trauma. Participants completed multiple clinical questionnaires to assess their levels of childhood trauma and anxiety proneness. The Western Cape Department of Education gave permission to access secondary schools. Participants gave written assent for participation in the study and their parents and (or) guardians provided written consent. Ethical approval was provided by the Health Research Ethics Committee of Stellenbosch University.

The current research focuses on a study sample consisting of a subset of participants from the larger parent study investigating both AP and AS (Martin et al., 2014). The DNA methylation profiles of these samples were investigated by using the Illumina 450K BeadChip (n = 46) and EpiTYPER MassARRAY technology (n = 63). All participants self-identified as adolescents of either South African Coloured or Black ethnicity. These samples were selected based on their self-reported levels of childhood trauma (as measured using the childhood trauma questionnaire (CTQ)) (Bernstein & Fink, 1998) and levels of AP (as measured using AS and TA scores), during initial recruitment, and were subdivided into the following four groups: i) healthy adolescents with low levels of self-reported childhood maltreatment and low levels of AP; ii) healthy adolescents with low levels of self-reported childhood maltreatment and high levels of AP; iii) healthy adolescents with high levels of self-reported childhood maltreatment and low levels of AP; and iv) healthy adolescents with high levels of self-reported childhood maltreatment and high levels of AP. Group status was determined by selecting those participants who fell within the upper 66th and lower 33rd (i.e. high and low) percentile for each variable of interest i.e. CTQ and AP scores. Thereafter, group status was re-calculated based on initial the CTQ status and by using the 50th percentile of AP score during second recruitment.

Groups were matched on age, ethnicity, gender and educational status as closely as possible. The study sample is summarised in Table 3.

Table 3: Sample statistics for each method of DNA-methylation investigation

Method of Investigation	Group Status	n	Percent	Valid Percent	Cumulative Percent
Illumina 450K Beadchip Array	Low CTQ and Low AP	16	34.8	34.8	34.8
	Low CTQ and High AP	9	19.6	19.6	55.4
	High CTQ and Low AP	15	32.6	32.6	87
	High CTQ and High AP	6	13	13	100
	Total	46	100	100	
EpiTYPER MassARRAY System	Low CTQ and Low AP	25	39.7	39.7	39.7
	Low CTQ and High AP	17	27	27	66.7
	High CTQ and Low AP	12	19	19	85.7
	High CTQ and High AP	9	14.3	14.3	100
	Total	63	100	100	

Abbreviations: DNA – deoxyribonucleic acid; CTQ – childhood trauma questionnaire; AP – anxiety proneness, n – number.

Footnote: With the exception of 3 samples, all samples used that were subjected to 450K BeadChip analysis were also subjected to analysis with the EpiTYPER MassARRAY system.

All participants underwent interview-based neuropsychiatric assessments, neurocognitive assessments and brief medical examinations, in order to exclude participants with clinical conditions that may influence the results. Peripheral whole blood samples were collected from each participant and stored in EDTA vacutainer tubes (Kendon Medical Supplies, Cape Town, SA) for subsequent DNA extraction and downstream genetic and epigenetic application.

2.2 Clinical Questionnaires

The following tests were administered during initial recruitment, and validated during second recruitment, to the study sample to confirm participant group status:

(1) The *Childhood Trauma Questionnaire* (CTQ) is a 28-item self-report measure used to identify individuals with histories of childhood trauma with regard to five scales: physical abuse, sexual abuse, emotional abuse, physical neglect and emotional neglect. The questionnaire also includes a minimisation scale to identify participants that may underreport traumatic events (Bernstein & Fink, 1998).

(2) The *State-Trait Anxiety Inventory* (STAI) is a commonly used introspective, 40-item self-report measure of state and trait anxiety, which are all rated on a 4-point scale, for diagnosis of anxiety and to distinguish clinical anxiety from depressive syndromes (Spielberger, 2010).

(3) The *Childhood Anxiety Sensitivity Index* (CASI) is an 18-item self-report measure to assess anxiety sensitivity in children and adolescents between 6 to 17 years. Several studies have reported good consistency for the test (Silverman et al., 1991).

2.3 Genome-Wide DNA Methylation Analysis

DNA was extracted from peripheral whole blood using the standard phenol/chloroform method and subsequently quantified using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Walton, MA, USA). DNA samples were then diluted to a concentration of approximately 50 ng/ul, and re-quantified with the NanoDrop 2000c spectrophotometer to ensure accurate normalisation of DNA quantity. Thereafter, approximately 1 ug of DNA was subjected to bisulfite treatment using the EZ DNA Methylation Lightning Kit (Zymo Research, Irving, CA, USA) in order to maintain all methylated cytosines and convert all unmethylated cytosines within the samples. Efficiency of the bisulfite conversion was monitored by bisulfite-converting three controls in parallel with sample DNA - a total methylated control (enzymatically methylated Jurkat genomic DNA), an intermediate-methylated control (genomic Jurkat DNA) and a non-methylated control (whole-genome amplified genomic DNA).

The HumanMethylation450K DNA analysis BeadChip array (Illumina, San Diego, CA) was selected for whole-genome methylation analysis. The platform allows for high-throughput, multiplex methylation analysis on a single chip, requiring minimal DNA input and minimizing risk of carry-over contamination. This platform determines the methylation levels for more than 450 000 pre-selected, but highly informative CpG sites, targeting sites within promoter regions, 5' untranslated regions (UTRs), first exons, gene bodies and 3'-UTRs in order to provide a broad and comprehensive view of the methylation state of the entire genome. Following successful bisulfite conversion, bisulfite-converted DNA was then subjected to whole-genome methylation analysis according to the Manual Protocol for the Illumina Infinium HD Methylation Assay.

Briefly, bisulfite-converted DNA was subjected to denaturation and neutralization, followed by whole-genome isothermal amplification and endpoint fragmentation using specialised enzymes to prevent over-fragmentation. Thereafter, the DNA was purified using isopropanol and re-suspended in hybridization buffer. The re-suspended DNA was then heat-denatured and hybridized to the HumanMethylation450K BeadChip (Illumina Inc., San Diego, CA) in a hybridization oven for approximately 23 hours. DNA samples were hybridised to the Bead Chip without knowledge of group status. The Bead Chips were then washed with buffer, labelled with nucleotides at the end of the probes and then stained using fluorescent marker antibodies to the incorporated nucleotides. Following a second wash step, coating and drying, fluorescent images were then obtained using the iScan Control Software v.3.2.45. Resulting images were background-normalised using Illumina BeadStudio software and analysed using GenomeStudio methylation software. The level of methylation at each locus was determined using the Illumina HiScan scanner (Illumina Inc., San Diego, CA). Ten samples were assayed in duplicate, to measure reproducibility.

2.4 EpiTYPER MassARRAY Verification

Upon acquisition of the processed Illumina 450K BeadChip data, CpG sites of interest were filtered, and selected for EpiTYPER MassARRAY verification, based on the following criteria, in order of importance:

i) P-values below the threshold of 0.05

ii) Log2 - fold change

iii) Expression within the CNS

iv) Previous scientific literature

Due to the exploratory nature of the study, unadjusted p-values below the threshold of p = 0.05 were used, as multiple testing correction lead to no CpG sites retaining significance, and these CpG sites thus represent the most appropriate candidates for investigation. Using the UCSC Genome Browser (www.genome.ucsc.edu) (Kent et al., 2002), CpG sites of interest, as selected from the Illumina 450K BeadChip, were used as proxy to identify CpG islands of interest for verification using the EpiTYPER MassARRAY® platform (Sequenom Inc., San Diego, CA, USA). CpG islands were defined as gene regions larger than 100 bp, with GC content greater than 50% and Observed/Expected ratios greater than 0.6. Additionally, previously investigated CpG islands, within five candidate genes, *BDNF, COMT, FKBP5, NR3C1* and *SLC6A4,* were selected for analysis using the same platform. This was done in order to provide a more accurate representation of the aforementioned genes' overall methylation status. Notably, the Illumina 450K Beadchip does not include any CpG sites in the promoter regions of *SLC6A4* or *BDNF* (the islands of which are situated in these regions), and it does not include *FKBP5* and *NR3C1* CpG sites that have previously been examined in literature.

The Sequenom EpiTYPER system is a highly reliable and quantitative technology for determining the density of methylated cytosines across specific genomic loci by utilising base-specific cleavage followed by MALDI-TOF mass spectrometry in which the size ratio of the cleaved products provides quantitative methylation estimates for CpG sites within a target region. EpiTYPER MassARRAY methylation analysis was performed by Roswell Park Cancer Institute (Buffalo, NY, USA). Polymerase chain reaction (PCR), reverse transcription, cleavage and mass spectrometry were performed in duplicate and in conjunction with high-, low- and 50%-methylated human genomic DNA controls (EpigenDx, Hopkinton, MA, USA) according to the EpiTYPER protocol.

Sodium Bisulfite treatment

500 µg genomic DNA per sample was subjected to bisulfite treatment using the EZ DNA Methylation Kit from Zymo Research (Zymo Research, Irving, CA, USA) as per manufacturer's instructions with the singular exception that water, and not elution buffer, was used in the final step. DNA was then eluted to a concentration of 10 ng/ul. The bisulfite treatment produces methylation-dependent sequence variations of C to T. These C/T variations appear as G/A changes on the reverse strand, which result in a mass difference of 16 Daltons per CpG site. This mass difference is easily detected by the MassARRAY system and thereafter readily apparent in the signal pattern.

Amplification, SAP-treatment and T-reaction

Bisulfite-treated DNA was then subjected to PCR amplification using specially designed primers which flank the CpG regions and (or) islands of interest within each gene. MethPrimer (www.urogene.org/methprimer) was utilized to design the primers. Each reverse primer was tagged with a T7-promotor tag for *in vitro* transcription (5'-cagtaatacgactcactatagggagaaggct-3') and the forward primer were tagged with a 10-mer to balance the PCR (5'-aggaagagag-3'). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). Primer sequences, forward and reverse, used to verify CpG sites of interest acquired from the genome-wide analysis as well as the CpG islands within the candidate genes are summarised in Table 4 and Table 5, respectively. Due to the CG-rich nature of the *NR3C1* exonic region targeted, appropriate primers to analyse the full promoter region could not be synthesised and thus only a portion of the exon was analysed.

Table 4: Forward and Reverse Primers used for genome-wide analysis verification

CpG Probe ID	Gene	Gene Region	Forward Primer (5'- 3')	Reverse Primer (5' – 3')	Product Size (bp)
CG20608783	*BAI2*	Intron	TGTAGGAAGAGTTAT AAGTTTTTTGATA	AAAAATAACCTAAC CCAATACTTTC	304
CG05483731	*GALNT9*	Intron	TTGGGGAAAAGATT AGAATAGATA	CCAAATTATTTTCCA AACAACTAC	258
CG24445388	*PRKCZ*	Intron	TTTGTATTTAAAAGA	AAACCTTCCTCTCTC	183

			ATTGGAAATAGAT	TACCTAACC	
CG01263386	*SLC8A2*	Exon	TAGGAGTTAGAGGTT TAGGAAGAG	AAAAATCTTAAACC CACACTACCC	454
CG00030117	*STEAP3*	Intron	TTTATAATTGGATTTT AAAGGGTA	ACAAAAAAATTCTT ACAAAAAAAA	248

Abbreviations: CpG – cytosine-guanine dinucleotide; ID – identity; bp – base pairs; ID – identification; BAI2 – brain-specific angiogenesis inhibitor 2; GALNT9 – N-acetyl galactosaminyl transferase 9; PRKCZ – protein kinase C zeta; SLC8A2 – solute carrier family 8 member 2; STEAP3 – six-transmembrane epithelial antigen of the prostate 3.

Table 5: Forward and reverse primers used for candidate gene analysis

Gene	Gene Region	Forward Primer (5'- 3')	Reverse Primer (5' – 3')	Product Size (bp)
BDNF (Island 1)	Promoter	ATGGGTTATGGTTTT TTAAATGTGAGT	AAAACTTCCTAAAAA AACCCCCTC	265
BDNF (Island 2)	Promoter	TTTGTTTTTGGGAAT ATTTGTATG	TATTAACTCACATTTA AAAAACCATAAC	216
BDNF (Island 3)	Promoter	TTTTAGAGAAAGTGA GGAGTTTTAGGGT	TAATAAACAACAAAT TCCCCAAC	205
BDNF (Island 4)	Promoter	GAGGGGGTTTTTTTA GGAAGTTTT	CATTCATCCCTAATTC TTCTACTCTACT	394
BDNF (Island 5)	Promoter/ Exon 1	TTGGGGAATTTGTTG TTTATTAG	AAATATTCCCAAAAA CAAATCACTTACT	291
COMT	Promoter	TTAGTTTTTTTATTTG GGAAGGGG	AACAACCCTAACTAC CCCAAAAAC	341
FKBP5 (Region 1)	Intron 7	TTAAGGTAATTGATA AATTTTTTTT	TTTATTCCTAAACTTA TTCTTTTTATAC	306
FKBP5 (Region 2)	Intron 7	GTGATTTTTGTGAAG GGTATAATT	TTATCTCTTACCTCCA ACACTACTACTA	300
NR3C1	Exon 6	GAGGGTGGGTTTTGT	CCCTCTAAAAAAACT	183

		TTTGTAAT	TCAA	
SLC6A4 (Island 1)	Promoter	AAAGATTAAATATAA ATTATGGGTTGAA	AAAAATCAAACCATA TAAAAACCC	541
SLC6A4 (Island 2)	Promoter	GGGTTTTTATATGGT TTGATTTTTAGAT	CCTACTCCTTTATACA ACCTCCCC	333
SLC6A4 (Island 3)	Promoter	GGGGGAGGTTGTAT AAAGGAGTAG	CTAAACTAAACTCAC ATCCCAACC	218

Abbreviations: bp – base pairs; BDNF – brain-derived neurotrophic factor; COMT – catechol-O-methylatransferas; FKBP5 – FK506 binding protein 5; NR3C1 – nuclear receptor subfamily 3 group C member 1; SLC6A4 – solute carrier family 6 member 4.

Amplification of 1 µL of bisulfite treated DNA (10 ng/µL) was performed using HotStar Taq Polymerase (Qiagen, Valencia, CA, USA) in a 10 µL reaction volume using a 384-well microtiter plate. PCR primers were used at a 200 nM final concentration. PCR amplification was performed using a C1000 Touch Thermal Cycler (BioRad, Hercules, CA, USA) with the following parameters set out in Table 6.

Table 6: Detailed Amplification Cycle

	Primer Activation	**DNA Denaturation**	**Primer Annealing**	**Extension**	**Final Extension**
Temperature	94°C	94°C	Δ°C	72°C	72°C
Time	15 min	20 sec	30 sec	60 sec	3 min
Cycles	1	45			1

Footnote: Δ - Annealing temperatures were determined with gradient PCRs using methylated and unmethylated control DNA

Abbrevations: DNA – deoxyribonucleic acid

The PCR products were then subjected to 4 µl Shrimp Alkaline Phosphotase (Shrimp Alkaline Phosphatase, Sequenom/Agena, San Diego, CA, USA) treatment for 20 min at 37°C to remove

excess dNTPs, followed by a 5-minute incubation at 85°C. The T reaction was then performed on the reverse strand by preparing a transcription/RNase A cocktail. A fraction of the transcription/RNase A cocktail (5 µL) (Sequenom/Agena, San Diego, CA, USA) and 2.5 µL of the processed sample was then combined into a new microtiter plate and thereafter incubated at 37°C for 3 hours. Following incubation, 18 ul of double distilled water was then added to each sample. Additionally, 6 mg of Clean Resin (Sequenom/Agena, San Diego, CA, USA) was also added to the samples. The samples were then rotated for 15 minutes and centrifuged for 15 minutes at 3,000 x g.

Mass Spectrometry

A fraction of the treated product (10-40 nl) was then spotted to the appropriate location on a 384-pad SpectroCHIP II (Sequenom/Agena, San Diego, CA, USA) using a Sequenom MassARRAY Nanodispenser (Sequenom/Agena, San Diego, CA, USA). A MassARRAY Analyzer Compact MALDI-TOF MS (Sequenom/Agena, San Diego, CA, USA) was then used for data acquisition from the SpectroCHIP. Each spot is ionized 50x per second at five different rasters with all resultant methylation calls being analyzed by the EpiTyper software v1.2 (Sequenom/Agena, San Diego, CA, USA).

2.5 Statistical Analysis

All calculations and statistical analysis were done using the freely available R statistical program (R Studio) (Version 3.2.5).

2.5.1 Genome-Wide Methylation Statistical Analysis

The genome-wide methylation statistical analysis aimed to identify differentially methylated genes by comparing the following groups against each other:

Table 7: Statistical comparisons conducted from genome-wide methylation data	
High Levels of AP + High levels of CTQ (n = 6)	High Levels of AP + Low levels of CTQ (n = 9)
Low Levels of AP + High levels of CTQ (n = 15)	Low Levels of AP + Low levels of CTQ (n = 16)

High Levels of AP + Low levels of CTQ (n = 9)	Low Levels of AP + Low levels of CTQ (n = 16)
High Levels of AP + High levels of CTQ (n = 6)	Low Levels of AP + High levels of CTQ (n = 15)
High Levels of AP + High levels of CTQ (n = 6)	Low Levels of AP + Low levels of CTQ (n =16)
High Levels of AP + Low levels of CTQ (n = 9)	Low Levels of AP + High levels of CTQ (n = 15)
High Levels of AP (n = 15)	Low Levels of AP (n = 31)
High levels of CTQ (n = 21)	Low levels of CTQ (n = 25)
Abbreviations: AP – anxiety proneness CTQ – childhood trauma questionnaire	

Statistical analysis of the Illumina HumanMethylation450K BeadChip array data was performed by NXT-Dx (Ghent, Belgium). Methylation values from the HumanMethylation450K BeadChip array are provided in outputs known as β-values. Following normalisation using the subset-quantile within array normalization (SWAN) method from the *minfi* Bioconductor package (Maksimovic et al., 2012), these β-values were then used to generate M-values using the following conversion:

$$Beta_i = \frac{2^{M_i}}{2^{M_i}+1}; M_i = \log_2\left(\frac{Beta_i}{1-Beta_i}\right)$$

Although the β-values have more intuitive biological interpretations, the M-values are more statistically valid for the differential analysis of methylation levels. The Limma package (http://bioconductor.org/packages/release/bioc/html/limma.html) was used to generate linear models for each probe on the array. The Limma package allows for the use of linear models to analyse the entire experiment as an integrated whole rather than making piece-meal comparisons between pairs of treatments. Analysing the data as a whole also allows modelling of correlations that may exist between samples due to repeated measures or other causes. This kind of analysis would not be feasible were the data partitioned into subsets and analysed as a series of pairwise comparisons. Additionally, the use of the Limma package allowed for the

adjustment of batch effects. A number of possible confounders were included in the model such as age; ethnicity; gender; socioeconomic status; nicotine, cannabis and alcohol use and weight. A 2 × 2 factorial design interaction model was used. Factorial designs are those where more than one experimental dimension is being varied and each combination of variable conditions are observed. The two experimental dimensions in the study were levels of AP (high/low) and level of childhood trauma (high/low). Statistical significance values (p-values) were generated by using t-tests and adjusted for multiple testing using the Benjamini and Hochberg test (Benjamini and Hochberg, 1995). Statistical threshold was set at 0.05. In addition, log2-fold change was also calculated to represent the value of contrast between two comparative groups. Finally, the Limma package was also used to generate heatmaps for all comparisons conducted.

2.5.2 EpiTYPER MassARRAY Statistical Analysis

Methylation values (measured as a %) at all investigated CpG sites as analysed by the EpiTYPER MassARRAY system were acquired in duplicate and following calculation of significant differences, and standard deviations, these values were averaged in order to acquire single methylation values. Data was sorted into the four categorical variables: Low CTQ – Low AP (n = 25); Low CTQ – High AP (n = 17); High CTQ – Low AP (n = 12) and High CTQ – High AP (n = 9). The data was also manually investigated and the amount of resolved and unresolved CpG sites within a given region or island was calculated.

Methylation levels of the CpG islands of interest were calculated by averaging the methylation levels of the individual CpG sites within the particular island and data distribution plots were generated thereafter to check the normalcy of the data. Cases where no CpG site within a given region or island could be resolved, were excluded from further analysis in that region. Correlation analysis was performed between the individual CpG sites within a given region or island of interest using the R package “corrplot” (Wei, T., 2013). This was done in order to conclude whether methylation across the region was uniform enough to justify continuing analysis on a given region as a whole. As data had already been sorted into the aforementioned categorical variables, following correlation analysis, these four categorical variables were then

ordered in the same sequence, as previously mentioned, in order to conduct trend tests on the data. Trend tests were conducted to detect linear correlation between the dependant variable (percentage methylation in a given CpG region or island) and a set order of independent categorical variables (group status). The trend test allows for a statistically powerful test given the small sample numbers within each categorical variable. Trend tests were conducted using the generalised linear model (logistic regressions), and during all conducted tests, all confounders (age, ethnicity, gender, socioeconomic status, nicotine, cannabis and alcohol use and weight) included during the genome-wide methylation analysis were included in the model. Additionally, unpaired t-tests were also conducted to test for associations between these confounders and overall methylation within a given CpG region or island of interest. In order to investigate whether significant trends were of an upward or downward nature; generalised linear models were plotted as boxplots in order to illustrate significant trends.

2.6 Pathway Analysis

Using Ingenuity Pathway Analysis (IPA) (Version 1.07), functional assignment and pathway analysis was conducted on the results obtained from the Illumina 450K BeadChip array in order to identify underlying differentially methylated pathways that may be associated with childhood trauma experience and (or) AP. BeadChip results were filtered for the retention of unadjusted p-values below a threshold of 0.05 as well as fold changes exceeding 2. This filtration allowed for sufficient amount of genes for input. Unadjusted p-values were used as multiple testing correction lead to no genes retaining significance. Multiple assessments were conducted including overrepresentation analysis of pathways assigned to the input genes, network construction between gene products and genes, functional assignment, disease associations and assessment of shared regulatory molecules of the input genes. Analysis filtration parameters were defined to draw information from the Ingenuity Knowledge Base (genes only). Additional filtration parameters were defined to include only results pertaining to *Homo sapiens* (to exclude any gene functions annotated through homology from other species) and to include only experimentally confirmed interactions, providing maximum confidence in the results obtained. Network construction was not constrained as exploration into related genes was desired, thus direct and indirect relationships were used. Direct interactions biased

construction towards protein-protein binding of two input gene products, whereas indirect interactions allowed for the connection of input genes that function in the same pathway but do not necessarily interact with each other.

3. RESULTS

3.1 Sample Characteristics

Study participants were recruited from secondary schools in Cape Town and surrounds. Along with undergoing whole blood extraction and clinical questionnaires assessment, nicotine, alcohol and cannabis use were also noted. Table 8 summarises the study sample's demographic characteristics and lifestyle factors. The predominantly black and female study sample consisted of adolescents with a mean age of 16 years and a mean weight of 63.07 kg.

Table 8: Demographic and lifestyle characteristics of study participants

	Low AP-Low CTQ (n = 25)	Low AP-High CTQ (n= 12)	High AP-Low CTQ (n = 17)	High AP-High CTQ (n = 9)
Gender (n)				
Female (%)	16 (64%)	8 (67%)	13 (76%)	5 (56%)
Male (%)	9 (36%)	4 (33%)	4 (24%)	4 (44%)
Ethnicity (n)				
Coloured (%)	7 (28%)	1 (8%)	4 (24%)	1 (11%)
Black (%)	18 (72%)	11 (92%)	13 (76%)	8 (89%)
Nicotine Use (n)				
Yes (%)	2 (8%)	0 (0%)	0 (0%)	1 (11%)
No (%)	23 (92%)	12 (100%)	17 (100%)	8 (89%)
Alcohol Use (n)				
Yes (%)	10 (40%)	3 (25%)	4 (24%)	2 (22%)
No (%)	15 (60%)	9 (75%)	13 (76%)	7 (78%)
Cannabis Use (n)				
Yes (%)	1 (4%)	0 (0%)	0 (0%)	1 (11%)
No (%)	24 (96%)	12 (100%)	17 (100%)	8 (89%)

Abbreviations: AP – anxiety proneness; CTQ – childhood trauma questionnaire ; n - number

3.2 Genome-Wide Methylation Analysis Results

The Illumina supplied HumanMethylation450K DNA analysis BeadChip array was used to analyse the methylation status of CpG sites across the whole genome. Following data analysis, during which several groups of interest were compared against each other (as summarised in Table 7) in a total of 46 participants, and subsequent Benjamini and Hochberg correction (Benjamini and Hochberg, 1995), none of the CpG sites analysed within the 450K BeadChip array reached significance levels of 0.05 or lower. However, a number of CpG sites were found to be significant when investigating the unadjusted significance values. Therefore, a filtration process was implemented to identify the top candidates for EpiTYPER MassARRAY verification. As the current study was only exploratory in nature, the unadjusted P-value was used as primary determinant for verification. Thereafter, log2-fold change was taken into account, as higher fold changes are more likely to represent biologically significant changes, as well as expression in the CNS, as anxiety disorders are primarily disorders of the brain, and previous scientific literature in the field. The top 6 differentially methylated genes from each comparison are summarised in Table 9. From the top candidates in each comparison, five candidate CpG sites of interest from across the comparisons were selected for EpiTYPER MassARRAY verification in order to represent all the Illumina 450K BeadChip analysis. These CpG sites were as follows: cg20608783, in *BAI2,* was differentially methylated between participants with low AP and low levels of CTQ compared to low AP and high levels of CTQ; cg05483731, in *GALNT9,* was differentially methylated between participants with low AP and low levels of CTQ compared to high AP and high levels of CTQ; cg24445388, in *PRKCZ,* was differentially methylated between participants with low AP and low levels of CTQ compared to low AP and high levels of CTQ; cg01263386, in *SLC8A2,* was differentially methylated between participants with high AP and low levels of CTQ compared to high AP and high levels of CTQ and cg00030117, in *STEAP3,* which was differentially methylated between participants with high AP and low levels of CTQ compared to high AP and high levels of CTQ. Following selection, these CpG sites of interest were used to identify CpG regions and (or) islands of interest within the five respective genes.

Table 9: Top 6 candidate CpG sites differentially methylated in each comparative analysis conducted using whole methylation data								
Probe Identification	**Log2 - Fold Change**	**P- Value**	**Adjusted P-Value**	**B**	**Chr**	**Gene**	**Accession Number**	**Genome Build**
High AP-High levels of CTQ vs. High AP-Low levels of CTQ								
cg18828681	-3.5170295	0.00094264	0.99999826	-3.5644408	14	ACOT6	NM_001037162	37
cg11723698	2.14423001	0.02187697	0.99999826	-4.1325016	1	ACOT7	NM_181864	37
cg01406776	-2.9386124	0.01525284	0.99999826	-4.0641195	4	ACOX3	NM_001101667	37
cg19601530	2.60453454	0.0001217	0.99999826	-3.2207573	4	AFAP1	NM_198595	37
cg24955955	-2.1580508	0.00907571	0.99999826	-3.970072	5	AHRR	NM_020731	37
cg07599136	-2.3428269	0.01783692	0.99999826	-4.0946985	5	AHRR	NM_020731	37
Low AP-High levels of CTQ vs. Low AP-Low levels of CTQ								
cg24445388	**-1.01756**	**5.13E-07**	**0.24895521**	**5.52657767**	**1**	**PRKCZ**	**NM_001033581**	**37**
cg21885868	-0.7637366	1.77E-06	0.85707158	4.51499869	21	POFUT2	NR_004858	37
cg03727754	-1.0908315	2.71E-06	0.99999587	4.16208707	12	CACNA1C	NM_001129844	37
cg16453474	-0.9613623	3.23E-06	0.99999587	4.01844301	5	C7	NM_000587	37
cg16719771	-0.5871189	3.39E-06	0.99999587	3.97787548	8	POTEA	NM_001005365	37
cg20608783	**-1.0592553**	**2.30E-05**	**0.9999956**	**1.2595901**	**1**	**BAI2**	**NM_001703**	**37**
High AP-High levels of CTQ vs. Low AP High-levels of CTQ								
cg00030117	**-4.754684**	**1.41E-06**	**0.68324594**	**-3.6102498**	**2**	**STEAP3**	**NM_001008410**	**37**
cg02718078	-2.1855048	3.27E-06	0.999999	-2.3137629	2	ARHGEF4	NM_032995	37
cg26555531	-0.7521708	6.77E-06	0.999999	-2.4380904	10	ADARB2	NM_018702	37
cg12062782	1.86134171	1.13E-05	0.999999	-2.5271611	11	RIN1	NM_004292	37
cg16437728	1.05367773	1.30E-05	0.999999	-2.553273	11	SYT9	NM_175733	37
cg01263386	**0.93188006**	**1.45E-05**	**0.999999**	**-2.5719293**	**19**	**SLC8A2**	**NM_015063**	**37**
High AP-Low levels of CTQ vs. Low AP-Low levels of CTQ								
cg11386709	-1.3397951	1.60E-06	0.77841848	1.86416083	6	EPB41L2	NM_001135554	37

cg09255732	-0.5511687	2.89E-06	0.99999851	1.56419252	1	COL16A1	NM_001856	37
cg05957546	-1.3039193	3.91E-06	0.99999851	1.3459209	11	OR5P2	NM_153444	37
cg21177626	-0.6324197	5.36E-06	0.99999851	1.24536413	6	FAM50B	NM_012135	37
cg27628370	-2.257305	7.42E-06	0.99999851	1.07504173	8	TMEM71	NM_001145153	37
cg23276617	0.78943754	9.57E-06	0.99999851	0.94154047	12	CLEC4A	NM_194448	37
Low AP-Low levels of CTQ vs. High AP-High levels of CTQ								
cg00030117	**-4.6559387**	**2.80E-06**	**0.99999726**	**0.87992985**	**2**	**STEAP3**	**NM_001008410**	**37**
cg27019712	-2.0720944	1.97E-05	0.99999726	1.41472634	17	PRKCA	NM_002737	37
cg26727538	-3.5801473	2.71E-05	0.99999726	1.62492069	19	CEACAM18	NM_001080405	37
cg23168520	-3.503333	5.63E-05	0.99999726	1.09911694	13	RASA3	NM_007368	37
cg03482458	-2.2841706	6.42E-05	0.99999726	1.00498841	22	CN5H6.4	NR_024009	37
cg18775025	-2.2962395	6.69E-05	0.99999726	0.97517731	10	STK32C	NM_173575	37
Low AP-High levels of CTQ vs. High AP-Low levels of CTQ								
cg10690152	5.36268042	4.48E-05	0.99999912	-3.5112139	6	DDAH2	NM_013974	37
cg21306988	-2.9762039	0.00024465	0.99999912	-2.7222046	13	LIG4	NM_206937	37
cg00013655	2.35829189	0.00038044	0.99999912	-3.6749718	16	PIGQ	NM_148920	37
cg15954263	-3.2333704	0.00045288	0.99999912	-2.8793782	3	FNDC3B	NM_001135095	37
cg01006048	-2.4828466	0.00143012	0.99999912	-3.1802814	10	GPR158	NM_020752	37
cg11144103	-2.0805192	0.00150589	0.99999912	-3.1940024	17	PTRF	NM_012232	37
High AP vs. Low AP								
cg05907082	0.82947166	8.18E-06	0.99999998	-3.1884217	12	POLE	NM_006231	37
cg15971542	-0.973549	9.11E-06	0.99999998	-3.2006745	12	PXN	NM_025157	37
cg00030117	**-5.2997872**	**1.18E-05**	**0.99999998**	**-4.0616462**	**2**	**STEAP3**	**NM_001008410**	**37**
cg07869405	-0.8914829	1.66E-05	0.99999998	-3.2697798	10	ADARB2	NM_018702	37
cg26707845	-0.7855206	2.36E-05	0.99999998	-3.3107839	11	ODZ4	NM_001098816	37
cg05483731	**-1.9281515**	**9.77E-05**	**0.9999956**	**0.32126647**	**12**	**GALNT9**	**NM_001122636**	**37**
High levels of CTQ vs. Low levels of CTQ								
cg24445388	**-1.6940758**	**1.48E-06**	**0.71645704**	**2.98815892**	**1**	**PRKCZ**	**NM_001033581**	**37**

cg02956660	-0.6798689	1.91E-06	0.92462843	2.83081254	1	HSPG2	NM_005529	37
cg01623261	-1.5814849	3.53E-06	0.9999956	2.44666256	17	BAHCC1	NM_001080519	37
cg00711916	-0.8810929	7.13E-06	0.9999956	2.00559747	2	CNNM4	NM_020184	37
cg00110675	-1.721729	2.33E-05	0.9999956	1.25130635	6	DDAH2	NM_013974	37
cg01263386	**1.20642297**	**3.27E-05**	**0.9999956**	**1.03355482**	**19**	**SLC8A2**	**NM_015063**	**37**

Abbreviations: Chr – Chromosome; AP – anxiety proneness; CTQ – childhood trauma questionnaire; CpG/CG - cytosine-guanine site; BAI2 – brain-angiotensin inhibitor 2; GALNT9 – N-acetyl galactosaminyl transferase 9; PRKCZ – protein kinase C zeta; SLC8A2 – solute carrier family 8 member 2; STEAP3 – six-transmembrane epithelial antigen of the prostate 3.

Footnote:

***Candidate genes selected for EpiTYPER MassARRAY verification are highlighted in bold.**

3.3 EpiTYPER MassARRAY Results

The UCSC Genome Browser (www.genome.ucsc.edu) was used to identify the region of the gene in which the CpG sites of interest were situated, as well as whether these regions were defined as CpG islands. The CpG sites, identified as differentially methylated in the 450K BeadChip analysis, found within the genes *SLC8A2*, *BAI2* and *GALNT9,* respectively, were situated in regions that are defined as CpG islands, while the CpG sites found within the genes *PRKCZ* and *STEAP3*, also as assessed from the 450K BeadChip analysis, were not situated within regions defined as CpG islands. Therefore, regarding the latter two genes, analysis was continued using only the region wherein the differentially methylated CpG site was situated, as identified by UCSC Genome Browser. Furthermore, five additional candidate CpG islands of interest, that have previously been investigated, in genes encoding *BDNF, COMT, NR3C1, FKBP5* and *SLC6A4,* were also analysed in order to provide a more accurate representation of the aforementioned genes' overall methylation status and to assess reproducibility when comparing with previous literature. The DNA sequences and CpG regions/islands targeted for verification (A – E) and candidate gene methylation analysis (F – J) are illustrated by Figure 2 and Figure 3, respectively.

A) *BAI2*

TGCAGGAAGAGCCATAAGCCTCCTGATATACGCCTGGCGTTCTCCGCCGATGTTCAACAAAGAAGCAAAA
TTCAATTTCTTCCTACAGGCACTAAATCCCAGGCCAGCCTCCAGGGCCTGTAACCTACCCGCGGCCCGGCT
GTCACTCGGACGGCTTCATTGACCGCCGTTAGAGGCCAAGCTAGCCTGGGAGAGAGGCCACAGCAAAGC
CACCGGGCACTTGCCGTGGATGGGCACGATGTCAGATGTCTCATTGGAGAAGCCAAGAAGGCCCCCATC
GAAAGTACTGGGTTAGGCTACTTTC

B) *GALNT9*

CTGGGGAAAAGACCAGAACAGACACGTCACCACCGAAGACGCCCAAGCAGCCATCAAGCACGTGCGCA
GGTGCCCCCCTCGGCGGCCATCAGGGAGCTGCCACTCGGAGCCACACGGGGGCCGCTGTCTGGCTACC
GGAAAGGCTGACGTGCAGCCGACGTACAAAAGCGGCGCCGGTGAGGGTCGCGGAGGGGCCGGAACG
CCCACGTGTGGCGGGTGGGGACGGGAGGTGGCGCAGCTGCTTGGAAAACAATCTGG

C) *PRKCZ*

TTTGCACTCAAAAGAACTGGAAATAGACTCAAACAGATTCTTGTACGCGAACCTTCACAGTGGCACCACT
CACAACAGCCAAGGGTGGGAACACTATAATGCCCCTTGGCAGGTGCACGCACAAACGTGGGGCCTCCA
CTGGACAACGGAACATTACCCGGCCAGGCAGAGAGAGGAAGGCCT

D) *SLC8A2*

CAGGAGCCAGAGGCCCAGGAAGAGCGCGGTGGTGGCGAGCTTGGGTCCGCGCGGGCCGCCCAGCTCG
CCGCCGATGTGCGGCCGGCGCCGGTACAGCAGCACGGCAATGCCCACGAAGGCGAAGACGGTGAAGA
GCGTGACGGAGAAGGCCAGCGTGCCAGTGCGCACCTCGAAGGGGCGGCCCTGCACCGCCCAGTACAC
GGCGGCCACAGACCAGGCGACGCCCAGGCCAAGGAACACGTTCACCGCGTTGGAGCCGGTCACGTTGC
CGATGGACGCGTCGGCGCACTGGTCCTGCAGCGCCGCCACCTTGCTGGCGAACGTGTCTGCGAGGCAG
AGACATACAGGTCGGAGGGGCTTTGCGCCGCCACCCACAGGGGCGGGCATCCGCCTGCCCCCTCCCAG
CCTTTCCCGGTCGCCTGCTTTCTGCGGGCAGTGTGGGCTCAAGACCCCT

E) *STEAP3*

CCCACAACTGGACCCCAAAGGGCACGGTGGGGAGGAAGAGGCAGAGCTCTGGTCTCCCTGTGGAGGG
AGTGCTGCTGTAAAGCGCCTTTCCCTCCACCCCCAACTCCCGCCGAGAACCCCCCCACCCCCGCAGGCAG
TTTGCTTTCCCCCTCCACAGCTTTCCTCCTACCCGAATACCCACCTCCCAGCTCTCTGCTCAGCCAGGGCCC
AAGGCCAGAGCCTCTCTTCTCTTGCAAGAACTTTCCTGT

F) *BDNF*

TTCCCAGAGAAAGTGAGGAGTTTCAGGGTACCCCTGTAAAAAAAAAAACTCAGCCTGCACACCGCTCCC
TTGCCCACTACGCAGGTCACACCTGGGGCTCACGGCGAAGCTGAATTCGGCTCGGGAGATTTCCCTGTC
GCCAGGTAAGAAAACCTTCGCCTTGTCAGGCTAGGGCGGGAAGACCGCTGGGGAACTTGTTGCTTATCA
GCGCCGGCAGACTACCGCTTTAATAATAATACCAGAAAAGCGCAGCAGGGAGGGGGTGGGGGGCGGC
AACGGCGACGACTAACCTCGCTGTTTACGTGACCGACTCACTGTGACTCTCTGGGTAAAAAAAGGAAAC
TTCTTAGAAAAGTTCGTGCCCCTCCCCCTCCCCCATCATGACTAAGGGTCTCCAGCCGATGAGGTCGTGA
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ACCAAGGCGAGCCACTAGTTGCCCACAGGAACCTGTGTAAGCCGAGCTCTCGAGCGGCTGAGTTGAAT
GAAGTTATGACTGCGTTTGCTCTAATGGGTTATGGCTTCCCAAATGTGAGTTAACAGCCCCGGTGCGCA
GCTAGGTCGGAAGGCGAGCCGCGGAGAGCGGAGCCGCGGGCCAAGGCAGGGACGGGAGGGGAGGC
GGCAGGTTGGGCGCTCCGCTGCTGCTCGGTGCTGCTTCCCCGGAGCCGCTTCCTCATCCTGCGGGGCT
GCCCCGCCGCCGGGGTGCCGGGGTGAGCGCGGCGGGAGCCAGGGCTGGGGTGGCGAACCTCGGAGG
GGGCTCTCCCAGGAAGCCCTGCGGTTAGCGCCCGCGCTTCCCCACCAGTGGCTGCGCAAGCTGGGAAG
CGAAGAGGTCACGTGTTGGCGGAAAGTAACTTGGAGGCGAAAGGATGAACTTTGAGAAGAGGAAAAA
AAGCACAATGAAACAAACTACCCCCTTGGAGTGTCTAATAGAGGGGGAGGCGGGAGGGGGGGCGGAG
GGGCGCCGCTCTCCGCGGGAGATGTGGGGACGACTCCGGAAATCTCGGGAAATAGGCAATAACTCGAG
CTTTGGCAACGTCCCCCAGCCTAGGGAGGCGCTAACGCAAGGTATCTTTCCACTGAAGCTGCCCTTTGCC
TACCCTCAGCCATCGCTAGCACAGCAGAGCAGAAGAACCAGGGATGAATGTAGACCAGTTGTTATGG

G) *COMT*

CCAGTTTCCCCACCTGGGAAGGGGGCTACTTGTGGCTAGAAGCAGCCCGGACTCCTGAGCAAGACTAG
ACCAAGAGGCCGGTATGTGGACACCCCCGCGTGGGCACCCCCACGGGGACACCCTGGCCACCGCCGCGG
CGGACACCCTCACGAGGACACCCCGGCCGCGCGGACACCTACCGCGGGGACGCCCCGACCCCATCCTAC
CTGCTGCGCCCCGCGCCGCGCCCCGCACCCCGCCCGCCACGGCCTGCGTCCGCCACCGGAAGCGCCCTC
CTAATCCCCGCAGCGCCACCGCCATTGCCGCCATCGTCGTGGGGCTTCTGGGGCAGCTAGGGCTGCC

H) *FKBP5*

CCAAGGCAACTGACAAATTCTCTCTCTTCTCTACTTGGAGAAGTATAAAAAAAAAATGGCTTCGGGTTAG
CTGCTTTCTTTCTTGTATCTCTGGTCACAGAGCCTAGTGGCCCTCGAGGACTTGCAGTTGGGATAACAAC
TTGGAGCCACAGTGCAGGCCTCTTCGTGACTCCTGTGAAGGGTACAATCCGTTCAGCTCTGAAAAGCTG
CACCCCACTCCCCCAAGGAGCCACTTGGCAGAACGTGAACCTTTCTGTCCTCAACCCAGGAAAAAAAAA
GTACAAAAAGAACAAGTCTAGGAACAAATAAGGGAACAAGTCTTGGATTCTACCCAAAAAAGTTAAAA
AAAAAAAAAAAAGCTGACACATAGGAACAAAATAAGAACACGGAGCTCCTTCGTTGTATATCAGCTGTG
CTATGTCAGTTGTTCTATTCTTCAGCAGCAGTGTTGGAGGCAAGAGACAA

I) *NR3C1*

GAGGGTGGGTTCTGCTTTGCAACTTCTCTCCCAGTGCGAGAGCGCGGCGGCGGCAGCTGAAGACCCGG
CCGCCCAGATGATGCGGTGGTGGGGGACCTGCCGGCACGCGACTCCCCCCGGGCCCAAAGTACGTATG
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AGCGCGGCCGAGACGCTGCGGCACCGTTTCCGTGCAACCCCGTAGCCCCTTTCGAAGTGACACACTTCA
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CCCGAGTGGGTCTGGAGCCGCGGAGCTGGGCGGGGGCGGAAGGAGGTAGCGAGAAAAGAAACTGG
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CCTTTTTCCTGGGGAGTTGGGGGCGGGGGGCGAAGCGCGGCGCACCGGGCGGGGCGGCCACGCCAGG
GGACGCGCGGGCGTGCAGGCGCCGTCGGGGCCGGGGTGGCGGGGCCCGCGCGGAGGGCGTGGGGGCA
GGGACCGCGCGGGCGCCCCTGCAGTT

J) *SLC6A4*

GAACTCTCCCTTTGCATAAAGCCACCTGCACGCGATGACAGCAAAGTAAAGATCAAACATAAACCATGG
GTTGAAACGAAAGCAAGCAACTCTTCCCAAAGCGCAGGACAGCACTTTGCTCAAGACCCTCTTTAAGGG
GTCTTTCACGGGTCCTCAAGAGGTTGCAAAGCCCCTGCAACAATAGACAAAGGAGCCCCCTACGCCGCC
CCACTTCGAGCACTCCACGTTCCTCGTCTCCCACTCTGGCCGGTCAGCTTCAGCTCTGGCTCTTGCTGAGC

GTGGAGGGCGCAGGGGCAGGATCCGGCGCCCACCGCTGGGGCGCATGCACCTCCTCGCCTCCTCGCAG
GGCGGTGGGGCGCAGCGGTTCTATCCCCCTCCCCGAGGCGGGGAAGAAGGTCTGGAAAGAAACGTGG

GTTCGAGGCGGAGAGGAAAAGCGGACCCACCTGCCAGGCTGCGCGGGGAGGCTGGTCCCGGGCTGGG
CAGGCGGGCTGGCCTCGCGCCCTCGAGGCACCCGGCGGCGCTGGCTGTGCGGAGGGGCGCCGGCGCG
CCGTATTTGTACCCGCGGGCCCTCACATGGTCTGATCTCTAGATAGCCGCCGCCAAAGAGCTCTTGAAGA
ATTTTTGCGTCACTTTGAGGCGAATAAACTTAATGCTTCCCCGCGGCCGCGGCTCCGCGCTCCCGCTGGA
TGGGGTTGCGCTCGCCAGGGAGGGGCCGCGCTACGGGGCGGGGTGCGCGCCCGACCCCAGAGCCAGG
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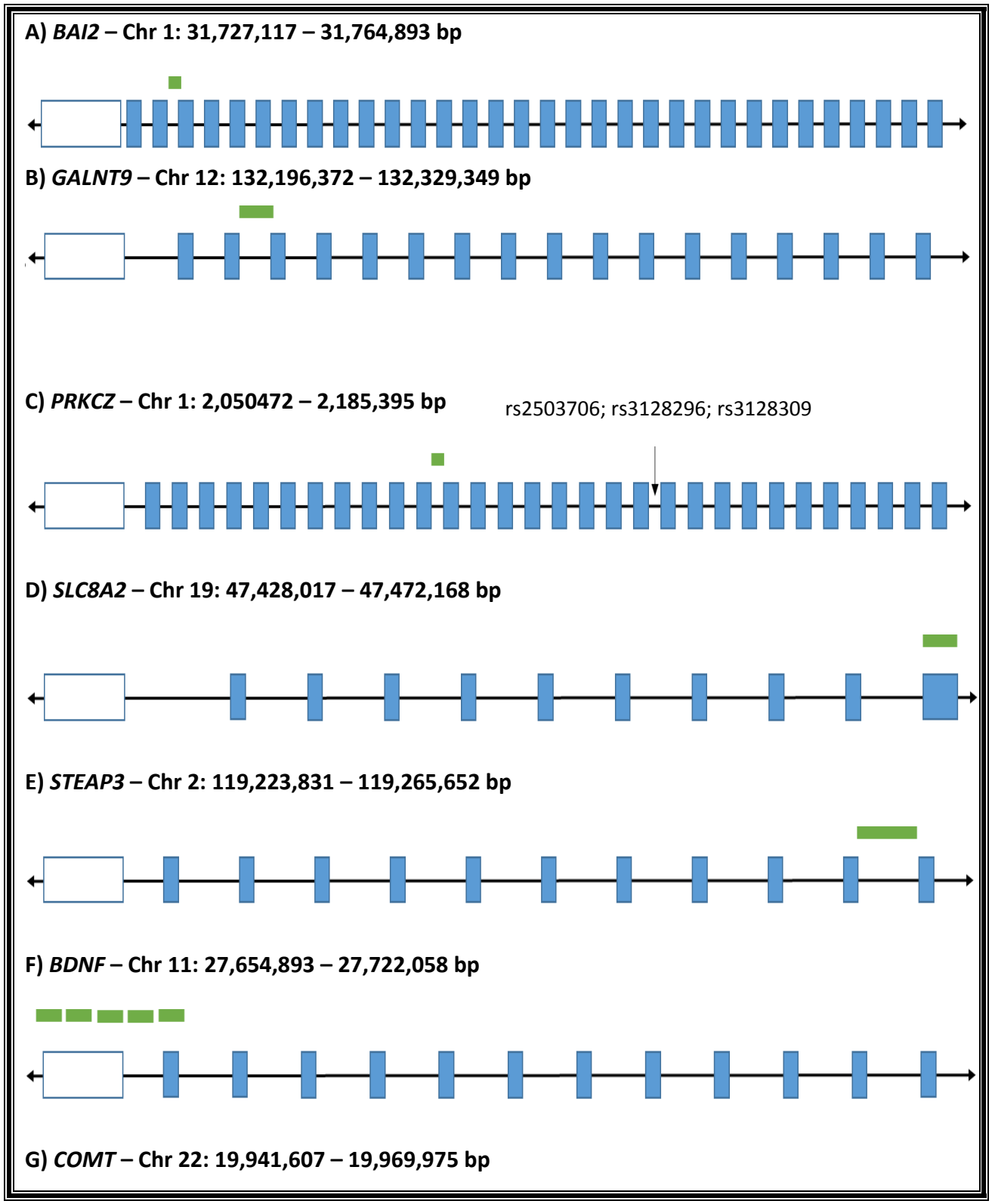
GGCAGGGCCGAGCGAGGAATCCTGACTTTCCTGCTCTTTAACTTTGCGGGAGGGGGGAGGCTGCACAA
AGGAGCAGGGTGTGCGCCTCCCCTGCCGCCCCGCGCCCACAGGACGGCACACAGGGGTCTGCTCGTGCC
GCTTTCTCTTGACCTCGGACACCTCCCCATCGTCCCCATCCCGAAAGCTGCTCTCTCTTTCTTCCTGGGACC
TAGCAGTACTCGATACCCATGCGTTTATCTGACTGACCGGCTGGGATGTGAGCTCAGCTTAGAGGCCCTT
GTCTAATTCCTCTCCGTCCCCAGAGTCTCACAATAAAGACTTCCACAATGAAGGAATGAAGAACTGGTTC
TCATTTTGTTAACCAGCTACAGTTCAAAGCT

Figure 2: DNA sequences targeted for verification from the genome-wide analysis (A – E) and for candidate gene methylation analysis (F – J)

Abbreviations: BAI2 – brain-angiotensin inhibitor 2; GALNT9 – N-acetyl galactosaminyl transferase 9; PRKCZ – protein kinase C zeta; SLC8A2 – solute carrier family 8 member 2; STEAP3 – six-transmembrane epithelial antigen of the prostate 3; BDNF – brain-derived neurotrophic factor; COMT – catechol-O-methyltransferase; FKBP5 – FK506 binding protein 5; NR3C1 – nuclear receptor subfamily 3 group C member 1; SLC6A4 – solute carrier family 6 member 4

Footnote:

CpG sites are highlighted in red; primers used for amplification are highlighted in blue, and green and purple in case of multiple primers; yellow regions indicate the original target region, targeted using all primers within a given region.



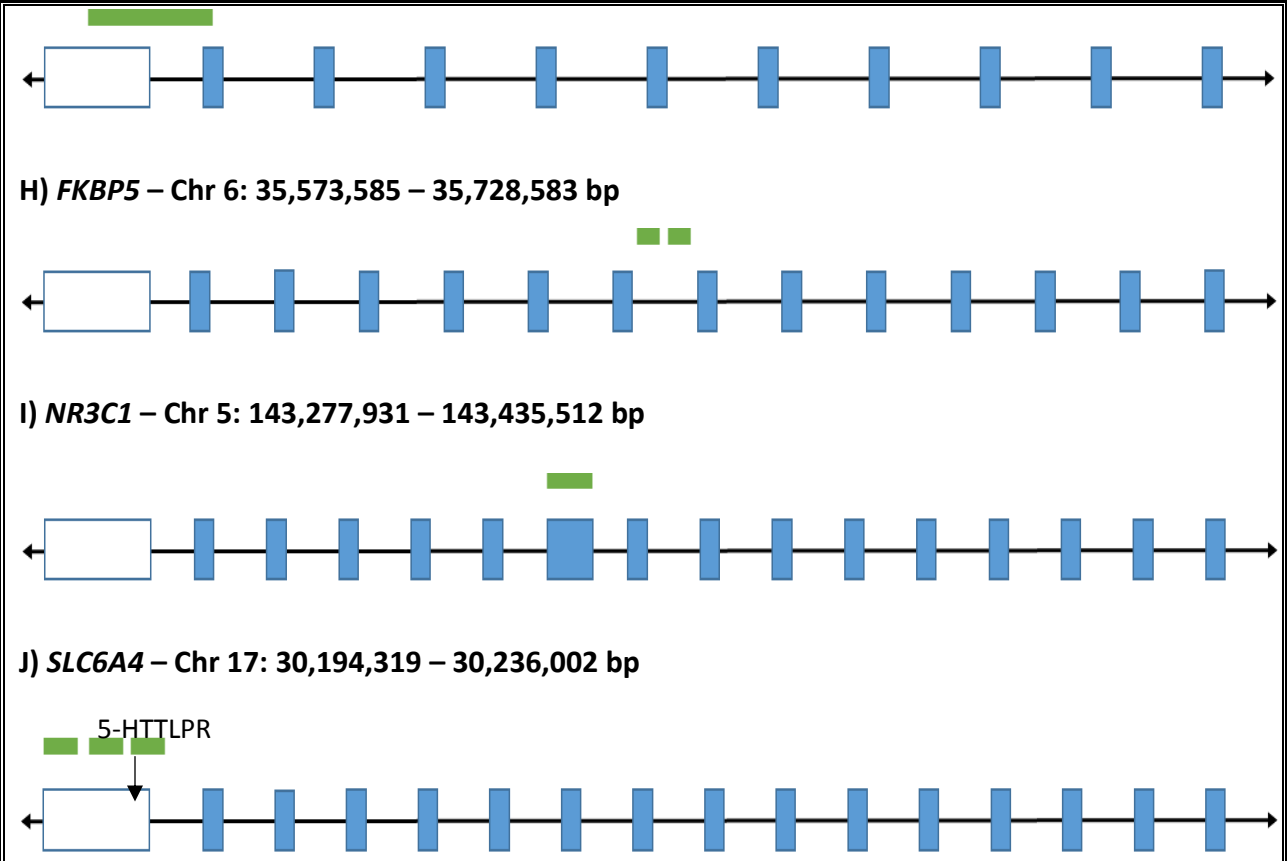


Figure 3: CpG regions and islands targeted for verification from the genome-wide analysis (A – E) and for candidate gene methylation analysis (F – J)

Abbreviations: BAI2 – brain-angiotensin inhibitor 2; GALNT9 – N-acetyl galactosaminyl transferase 9; PRKCZ – protein kinase C zeta; SLC8A2 – solute carrier family 8 member 2; STEAP3 – six-transmembrane epithelial antigen of the prostate 3; BDNF – brain-derived neurotrophic factor; COMT – catechol-O-methyltransferase; FKBP5 – FK506 binding protein 5; NR3C1 – nuclear receptor subfamily 3 group C member 1; SLC6A4 – solute carrier family 6 member 4; CpG – cytosine-guanine dinucleotide; bp – base pairs.

Footnotes:

Illustrations are not drawn on scale. All illustrations represent the sense strands. Notable SNPs (as referred to in discussion) are indicated by arrows.

Key: White boxes – promoter regions; blue boxes – exonic regions; green line – location of CpG regions and islands investigated

During EpiTyper verification, although the methylation of majority of the CpG sites under investigation within a given region or island could be resolved successfully; due to the CG-rich nature of the targeted regions, not all the CpG sites under investigation could be resolved. The level of successful resolution of CpG sites within a given region or island is illustrated in Figure 4.

Figure 4: Resolution of CpG Regions and Islands of Interest

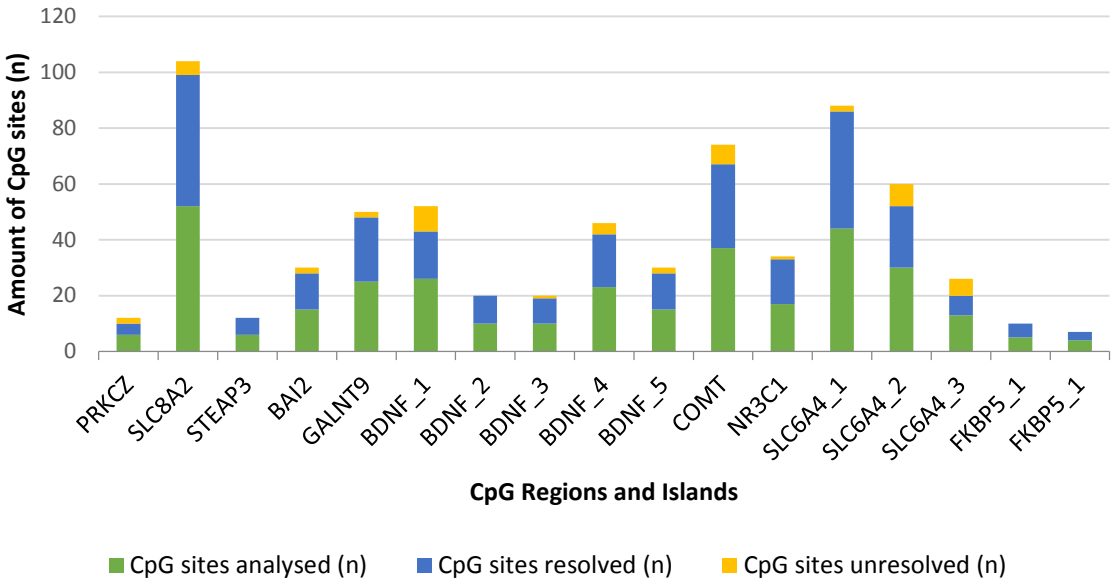
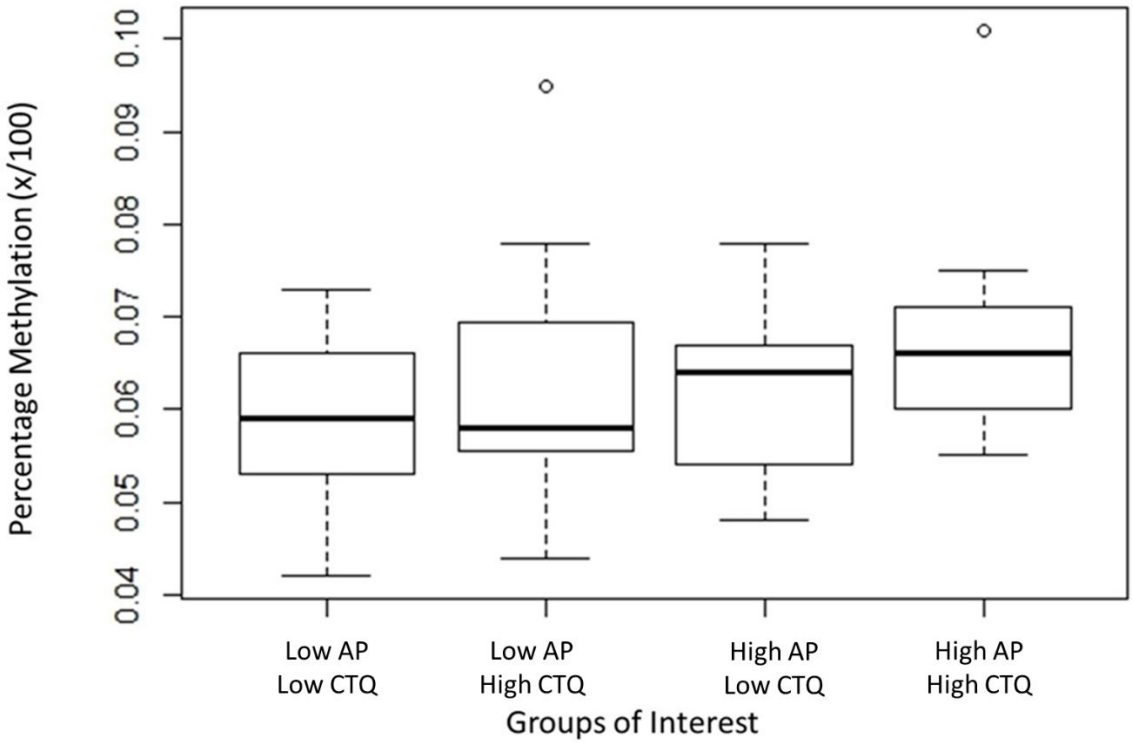


Figure 5: Graphic illustration of statistically significant trend observed in *SLC6A4_3*



Generalised linear models were used to conduct trend tests on all the investigated CpG regions and islands of interest to identify significant trends in methylation level across the four groups of interest (Low AP-Low levels of CTQ; Low AP-High levels of CTQ; High AP-Low levels of CTQ; High AP-High levels of CTQ), while controlling for age, gender, ethnicity, nicotine, cannabis and alcohol abuse, socioeconomic status and weight. None of the confounders (age, ethnicity, gender, socioeconomic status, nicotine, cannabis and alcohol use and weight) were associated with overall methylation within a given CpG island. A significant trend in methylation was observed for one of the CpG islands investigated, the 3rd island within the promoter region of *SLC6A4* ($p = 0.0461$). A boxplot was generated illustrating the trend and is displayed in Figure 5. No additional significant trends were observed. All trend tests conducted are summarised in Table 10.

Table 10: Trend test analysis for genome-wide analysis verification and candidate gene analysis										
CpG Island	**Statistical Parameters**	**Methylation Trend**	**Ethnicity**	**Age**	**Gender**	**Alcohol Use**	**Nicotine Use**	**Cannabis Use**	**SS**	**Weight**
Genome-Wide Verification Analysis										
BAI2	SE	10.66717931	0.373791	0.1181744	0.320271	0.33824	0.757477	0.905281	0.065673	0.012577
	T-value	0.855494056	-0.47809	0.5336686	0.3657	0.91399	0.234819	-0.40115	-1.05522	1.892938
	P-value	0.396358756	0.634671	0.5959355	0.716132	0.365109	0.815308	0.690018	0.296398	0.064161
GALNT9	SE	6.681400976	0.372734	0.1169937	0.317377	0.335569	0.741946	0.90208	0.063617	0.012496
	T-value	-1.25343112	-0.57869	0.5084378	-0.02982	1.124586	0.174859	-0.16925	-1.00085	1.960548
	P-value	0.21587841	0.565396	0.6133824	0.976326	0.266134	0.861897	0.866282	0.321718	0.055515
PRKCZ	SE	3.470478458	0.372028	0.1175617	0.313877	0.336246	0.754162	0.909698	0.064392	0.01254
	T-value	-1.02803473	-0.42779	0.4884615	0.147807	0.939786	-0.07903	-0.17088	-1.02761	1.819697
	P-value	0.30888122	0.670638	0.6273585	0.883089	0.351846	0.937324	0.865006	0.309078	0.074794
SLC8A2	SE	11.24613032	0.393131	0.1202983	0.322168	0.340447	0.767835	0.902742	0.064099	0.012599
	T-value	-0.93283645	-0.14659	0.5955919	0.561837	1.100804	0.346803	-0.25821	-0.8992	1.926582
	P-value	0.3555732	0.884068	0.5542459	0.576842	0.276472	0.730255	0.797352	0.373034	0.059964
STEAP3	SE	3.014511041	0.379466	0.1176259	0.316762	0.335905	0.744642	0.902498	0.063701	0.012552
	T-value	1.002520589	-0.61919	0.4705267	0.347394	0.980055	0.063214	-0.40987	-0.86051	1.81112
	P-value	0.320919447	0.538602	0.640025	0.729753	0.331778	0.949848	0.683649	0.393617	0.076132
Candidate Gene Analysis										
BDNF_1	SE	10.12358449	0.391446	0.1190123	0.316305	0.338908	0.770056	0.910444	0.064547	0.013185
	T-value	-0.35687038	-0.51208	0.503927	0.210766	1.003555	0.166592	-0.3557	-0.83573	1.875671
	P-value	0.722690602	0.610847	0.616526	0.833926	0.320425	0.868363	0.723565	0.407283	0.066545
BDNF_2	SE	9.215835622	0.379776	0.1185061	0.313956	0.336316	0.745728	0.922057	0.064379	0.012609
	T-value	-0.93060083	-0.6023	0.5808037	0.204973	0.987811	0.062026	-0.52116	-0.74529	1.76646

	P-value	0.356531653	0.549695	0.5639816	0.838426	0.328002	0.95079	0.604558	0.459586	0.083423
BDNF_3	SE	8.263011561	0.374715	0.1257377	0.318542	0.339614	0.741652	0.889307	0.063703	0.013084
	T-value	1.028886282	-0.2512	0.1036682	0.029701	1.329884	-0.16789	-0.41186	-0.81106	1.551804
	P-value	0.308799144	0.802754	0.9178737	0.976431	0.189975	0.867387	0.682311	0.421417	0.127417
BDNF_4	SE	11.3841215	0.40687	0.1364267	0.354713	0.36218	0.792972	0.923493	0.073669	0.013601
	T-value	0.49815572	-0.18159	0.5929985	0.690418	1.102808	-0.04558	-0.4089	-1.42766	1.411904
	P-value	0.620972941	0.856775	0.556362	0.493729	0.27639	0.963858	0.684688	0.160782	0.165345
BDNF_5	SE	13.54634505	0.397617	0.1199237	0.315729	0.336954	0.743967	0.910468	0.063293	0.012928
	T-value	1.004233363	-0.52874	0.3575529	-0.12611	1.273991	0.228353	-0.44169	-0.95644	1.704335
	P-value	0.320301967	0.599426	0.7222454	0.900174	0.208801	0.820342	0.660696	0.343644	0.094785
COMT	SE	11.92226409	0.375346	0.1186233	0.320517	0.340333	0.75344	0.917099	0.064227	0.012689
	T-value	0.421036838	-0.42029	0.4667512	0.132444	0.946033	0.126589	-0.38671	-0.86511	1.881959
	P-value	0.675532572	0.676072	0.6427054	0.895165	0.348682	0.899774	0.700611	0.391108	0.065668
FKBP5_1	SE	3.734482748	0.394971	0.1243717	0.357468	0.349917	0.738623	0.898442	0.070111	0.013226
	T-value	-1.01229851	-0.82428	0.6141243	-0.9113	1.026877	0.001553	-0.36358	-0.65904	2.353644
	P-value	0.317057845	0.41433	0.542369	0.367218	0.310221	0.998768	0.71795	0.513386	0.023231
FKBP5_2	SE	2.818439419	0.404371	0.1254816	0.329978	0.361658	0.761627	0.939786	0.07122	0.013018
	T-value	-0.45191082	-0.35295	0.2514172	0.56691	0.926043	0.158758	-0.309	-0.70721	1.496546
	P-value	0.653553188	0.725808	0.8026621	0.573656	0.359475	0.874586	0.758779	0.483165	0.141651
NR3C1	SE	41.28026489	0.377052	0.1175418	0.313313	0.337418	0.744083	0.899188	0.063724	0.012543
	T-value	1.040208109	-0.59766	0.4964231	0.219162	0.88855	0.118795	-0.35602	-0.82781	1.808601
	P-value	0.303247252	0.552767	0.6217714	0.827416	0.3785	0.905914	0.72332	0.41171	0.076528
SLC6A4_1	SE	7.890576919	0.379894	0.1239055	0.338409	0.342349	0.75696	0.912258	0.069444	0.012718
	T-value	-0.66796549	-0.50919	0.2774322	0.09055	1.061058	0.131033	-0.33664	-1.09492	1.850928
	P-value	0.507289836	0.612908	0.7826154	0.928219	0.293865	0.896286	0.737825	0.278904	0.070212
SLC6A4_2	SE	16.51704497	0.377782	0.1195528	0.314604	0.334208	0.745186	0.893419	0.063138	0.012661

	T-value	1.377311546	-0.13076	0.1696966	-0.00708	1.127195	-0.10124	-0.41489	-0.8986	1.583483
	P-value	0.174550694	0.896487	0.8659335	0.99438	0.26504	0.919767	0.680001	0.373172	0.119617
SLC6A4_3	SE	0.001359553	0.003621	0.0011446	0.003046	0.003293	0.007228	0.008744	0.000623	0.000126
	T-value	1.677299155	0.673057	1.3669793	-0.83332	0.319713	0.396408	0.022623	-0.20438	0.681118
	P-value	**0.0461 ***	0.50401	0.1777467	0.408626	0.750518	0.693491	0.982041	0.838885	0.498939

Abbreviations: CpG – cytosine-guanine site; SS – socioeconomic status; SE – standard error; BAI2 – brain-angiotensin inhibitor 2; GALNT9 – N-acetyl galactosaminyl transferase 9; PRKCZ – protein kinase C zeta; SLC8A2 – solute carrier family 8 member 2; STEAP3 – six-transmembrane epithelial antigen of the prostate 3; BDNF – brain-derived neurotrophic factor; COMT – catechol-O-methyltransferase; FKBP5 – FK506 binding protein 5; NR3C1 – nuclear receptor subfamily 3 group C member 1; SLC6A4 – solute carrier family 6 member 4

3.4 Pathway Analysis Results

IPA was used in order to conduct pathway analysis and identify patterns of differential methylation, from the data acquired from the 450K BeadChip array, enriched for biological functions, pathways and networks as well as disease associations. All data acquired from the comparisons conducted (as summarised in Table 7) were analysed, however, data was filtered to include only those results with fold changes exceeding 2 and unadjusted p-values below the threshold of 0.05 as these results are less likely to be statistical artefacts. Multiple shared, significantly enriched biological pathways and networks were observed. Similarly, multiple shared, significantly enriched disease associations were also found.

Functional Pathways

Among the functional pathways significantly enriched across the investigated comparative datasets; B-cell development and nicotinamide adenine dinucleotide (NAD) phosphorylation and dephosphorylation were enriched in the analysis comparing low to high levels of childhood trauma experience as well as low AP-low childhood trauma compared to high AP-high childhood trauma. Additionally, methylglyoxal degradation was also significantly enriched in the aforementioned datasets, as well as the analysis comparing low to high childhood trauma experience in high AP participants.

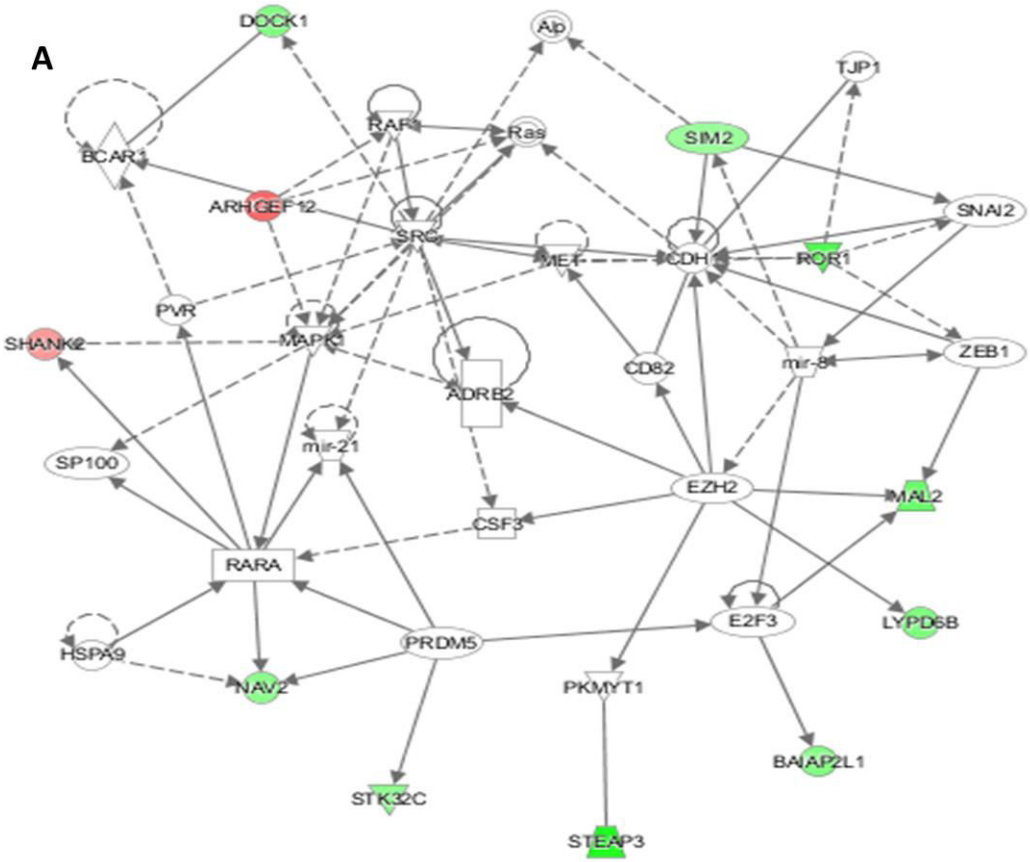
Disease Associations

Notably, two distinctive neuro-psychological disorders; hypersomnia and dyssomnia, were found to be significantly enriched in all the comparative analyses, with the exception of the comparison of low to high childhood trauma experience in low AP. However, these disease associations were found to be governed by only three common genes, including the major histocompatibility complex, DQ Beta 1 and DR Beta 1 genes (*HLA-DQB1* and *HLA-DRB1*) and the gamma-aminobutyric acid type A receptor Pi subunit gene (*GABRP*). Interestingly, metabolic disease association was found to be significantly enriched in the analyses comparing low to high childhood trauma experience and the comparison between low AP-high childhood trauma and high AP-low childhood trauma participants. Systemic autoimmune disease was also found to be

significantly enriched in the analysis comparing low to high childhood trauma experience. Both of the aforementioned disease associations were governed by several more differentially methylated genes as opposed to the hyper- and dyssomnia associations.

Functional Networks

Several shared functional networks were observed. These observed networks, generally included multiple cellular functions, including cellular assembly and organisation; cellular function and maintenance; cellular development and gene expression. Two example of these networks are illustrated in Figure 6 (A and B). Interestingly, the illustrated networks include the *STEAP3*, *HLA-DQB1* and *HLA-DRB1* genes, genes observed in both the disease association analysis as well as 450K BeadChip array analysis. The aforementioned network was observed in the analysis comparing low to high childhood trauma experience, the analysis comparing low AP-low childhood trauma to high AP-high childhood trauma and the analysis comparing low to high childhood trauma experience in high AP participants.



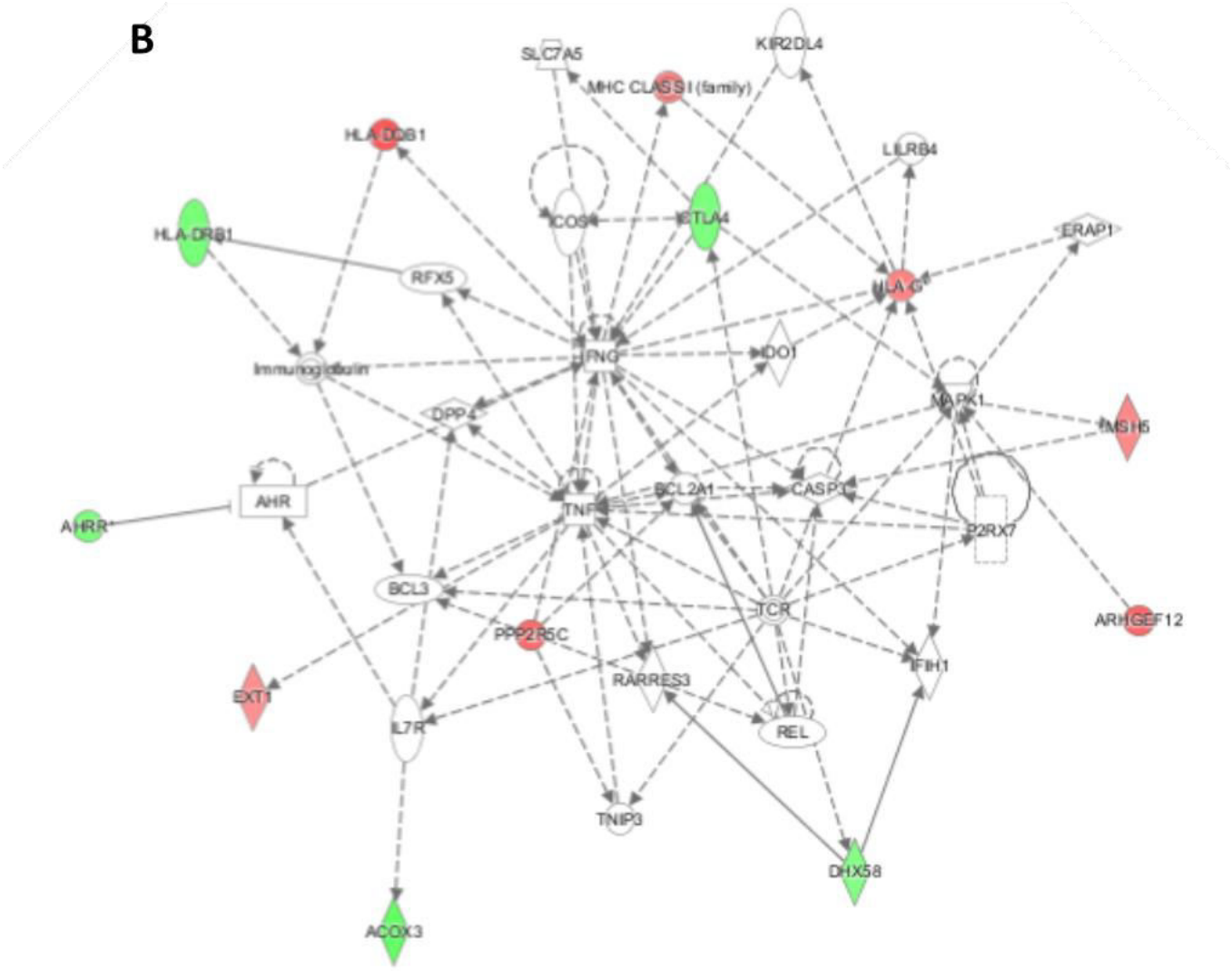


Figure 6: A) Functional Network enriched for cellular function and maintenance is comparison between Low AP-Low CTQ and High AP-Low CTQ. B) Functional Network enriched for cellular assembly and organisation is comparison between Low and High CTQ. Reduced methylation is indicated by green; increased DNA methylation is indicated by red; blank genes represent predicted genes. AP – anxiety proneness; CTQ – childhood trauma questionnaire; STEAP 3 – six-transmembrane epithelial antigen of the prostate 3; HLA-DQB1 – major histocompatibility complex, DQ Beta 1; HLA-DRB1 – major histocompatibility complex, DR Beta 1.

4. DISCUSSION

In the current study, we investigated the role that epigenetics, DNA methylation in particular, plays in the development of AP in South African adolescents. We aimed to conduct a genome-wide investigation into the role alterations in DNA methylation play in the development of AP in the context of differing levels of childhood trauma experienced. We verified the results from the genome-wide DNA methylation analysis by focusing on candidate regions of interest identified during the initial analysis. Additionally, analysis was extended to include investigation into a number of selected candidate genes that have previously been associated with anxiety disorders and, finally, pathway analysis was conducted to supplement the genome-wide analysis. Genome-wide alterations in DNA methylation were observed prior to correction, indicating an overall lack in statistical power, however a significant trend in methylation was observed in the promoter region of the *SLC6A4* gene associated with levels of childhood trauma exposure and anxiety proneness.

At the outset of the current study, the Illumina HumanMethylation450K Beadchip array was used to investigate global alterations in DNA methylation between four groups of interest namely; i) individuals who have high levels of anxiety proneness but have not experienced high levels of childhood trauma; ii) individuals who have high levels of anxiety proneness and have experienced high levels of childhood trauma; iii) individuals who have low levels of anxiety proneness but have experienced high levels of childhood trauma and iv) individuals who have low levels of anxiety proneness and who have not experienced high levels of childhood trauma. Thereafter, five candidate CpG sites were selected for verification using targeted techniques. These CpG sites were situated in the protein kinase C zeta gene (*PRKCZ*), the solute carrier family 8 member 2 gene (*SLC8A2*), the six-transmembrane epithelial antigen of the prostate 3 gene (*STEAP3*), the brain-specific angiogenesis inhibitor 2 gene (*BAI2*) and the N-acetyl galactosaminyl transferase 9 gene (*GALNT9*), respectively. Interestingly, although DNA methylation was measured in DNA extracted from whole blood, the majority of the top

differentially methylated genes as identified using the BeadChip array, although not significant after correction, are all involved in brain function. Although no statistically significant trends were observed upon verification of the genome-wide analysis, many of the genes under investigation have previously been associated with psychiatric conditions.

PRKCZ

The protein kinase C zeta gene, *PRKCZ*, codes for a protein that is highly expressed in the brain and functions as a serine/threonine kinase, involved in cell proliferation, differentiation and secretion (Hernandez et al., 2003). In murine studies, *PRKCZ*-knockout mice have displayed reduced anxiety-like behaviour (measured using the light-dark box and elevated plus maze tests) (Lee et al., 2013). Conversely, *PRKCZ* overexpression has been shown to improve long-term memory in flies and mice (Lee et al., 2013). A region in the gene has also been identified as a bipolar disorder susceptibility locus as a haplotype block, consisting of three intronic *PRKCZ* SNPs, has been associated with bipolar disorder development (Kandaswamy et al., 2012). Similarly, a *PRKCZ* haplotype has also been associated with the affectivity of antidepressant treatment and more specifically, antidepressant remission (Shi et al., 2012). It should be noted that the SNP's, as referred to above are not situated in the CpG region as investigated in the current study. Additional studies conducted in humans and cell lines have also associated *PRKCZ* methylation with non-psychiatric disease states that have been linked to psychological stress (Faulenbach et al., 2012; Moreno-Smith et al., 2010; Nikbakhsh et al., 2014; Smith et al., 2013), including type 2 Diabetes Mellitus (Zou et al., 2013) and cancer development (Seto and Andrulis, 2015), as well as lifestyle factors that have been linked to psychological stress (Azagba and Sharaf, 2011; Fluharty et al., 2016; Lykouras and Michopoulos, 2011; Sominsky and Spencer, 2014), including tobacco smoking (Steenaard et al., 2015) and obesity (Mansego et al., 2015).

SLC8A2

The solute carrier family 8 member 2 gene, *SLC8A2*, codes for the plasmamembrane Na+/Ca+ exchanger 2. This protein primarily functions as transport protein and regulator of cytosolic calcium concentration and has also been found to be significantly up-regulated during prenatal

development (Khananshvili, 2013). Predominantly and broadly expressed within the whole brain, *SLC8A2*-knockout mice have displayed enhanced long-term potentiation as well as enhanced performance in hippocampal-dependent learning and memory tasks (Jeon et al., 2003). Additionally, murine studies have also associated *SLC8A2* expression with stress-induced analgesia (Lisowski et al., 2012), cerebral ischemia (Morimoto et al., 2012), hypercalciuria and natriuresis (Gotoh et al., 2015), however *SLC8A2* research in humans is limited.

STEAP3

The six-transmembrane epithelial antigen of the prostate 3 gene, *STEAP3*, codes for a protein which primarily functions as a ferrireductase, reducing ferric iron to ferrous iron in endosomes, but is also involved in protein secretion, cell cycle and apoptosis regulation (Lambe et al., 2009). Like *PRKCZ*, the *STEAP3* gene has been linked to multiple disease states, including liver and lung cancer (Boelens et al., 2009; Gomes et al., 2012), congenital hypochromic anaemia (Grandchamp et al., 2011), Crohn's disease (Nielsen et al., 2009) and tumour growth (Isobe et al., 2011), as well as lifestyle factors, including smoking (Boelens et al., 2009; Nielsen et al., 2009) and the use of non-steroidal anti-inflammatory drugs (Slattery et al., 2015). Interestingly, impaired innate immunity-related inflammatory responses have been associated with *STEAP3* expression deficiency in bone marrow-derived macrophages (Zhang et al., 2012). Previously, abnormal and impaired immune and inflammatory responses have been associated with anxiety disorders and several related psychiatric disorders as well (Najjar et al., 2013; Salim et al., 2012; Zass et al., 2016).

BAI2

The brain-specific angiogenesis inhibitor 2 gene, *BAI2*, codes for a G protein-coupled receptor subtype. Highly expressed in the brain, this protein's primary function involves the inhibition of angiogenesis, however it has also been found to play a role in the regulation of synaptogenesis, dendritic spine formation and the promotion of nuclear factor activation in activated T-cells (Kee et al., 2002; Stephenson et al., 2014). Interestingly, murine studies have revealed that *BAI2*-knockout mice display resistance to depressive behaviour (as measured using the social defeat and tail suspension test) as well as increased neurogenesis within regions of the mouse

hippocampus via elevated expression of vascular endothelial growth factor (Okajima et al., 2011). Additionally, murine studies have also suggested a key role for differential *BAI2* expression during early and rapid brain development (Okajima et al., 2011) and differential *BAI2* expression has also been associated with tumorigenesis and tumor growth as measured in patients with highly advanced colorectal cancer (Kim et al., 2008).

GALNT9

Finally, the N-acetyl galactosaminyl transferase 9 gene, *GALNT9*, codes for an enzyme, which primarily functions as catalyst of O-linked glycosylation within the Golgi-apparatus and thus influences both cell adhesion as well as cell-cell communication (Toba et al., 2000). Like *PRKCZ, SLC8A2* and *BAI2*, this gene is pre-dominantly expressed within the whole brain and other CNS tissues (Toba et al., 2000), however studies investigating *GALNT9* has been largely limited to research in cancer (Berois et al., 2013; Pangeni et al., 2015). Methylation and subsequent silencing of *GALNT9* has been implicated in tumour metastasis from breast to brain (Pangeni et al., 2015), contributing to invasive potential of the tumour as well as poor relapse-free survival, while *GALNT9* expression has also been proposed as prognosis marker for neuroblastoma patients (Berois et al., 2013). Additionally, *GALNT9* has also been implicated in mitochondrial myopathy and sideroblastic anemia (Casas et al., 2004) as well as autism spectrum disorder (van der Zwaag et al., 2009).

Studies investigating DNA methylation in the aforementioned genes have been limited to cancer research, and thus far, no targeted DNA methylation studies have been conducted investigating any of these genes in anxiety disorders or any other related field. The lack of statistically significant trends in methylation observed in the gene regions investigated could be explained by a lack of statistical power, due to limited samples size, to observe statistically relevant trends. Additionally, the selection of candidate CpG sites for verification was based on the unadjusted p-value and could therefore represent false positives observed during the initial analysis. Recently, an increasing amount of epigenetic studies, employing the Illumina HumanMethylation450K Beadchip array, tend to pool CpG sites within the same gene during

statistical analysis and investigate differentially methylated regions instead of sites due to the likelihood of gathering false positives when only looking at individual CpG sites (Docherty et al., 2014; Jaffe et al., 2012; Slieker et al., 2013).

Following statistical analysis and subsequent verification of the Illumina HumanMethylation450K BeadChip array results, we decided to extend the DNA methylation investigation in a select amount of genes that have consistently been investigated and associated with anxiety disorders and/or related disorders. Although some of the CpG sites under investigation were included in the genome-wide analysis, in the extended DNA methylation analysis, CpG regions and islands were looked at specifically in order to provide a more accurate representation of the genes' overall methylation status. In addition the Illumina 450K Beadchip does not include any methylation sites in the promoter regions of *SLC6A4* or *BDNF*, and the sites it does include in *FKBP5* and *NR3C1* are not identical to the sites previously examined in the literature, making tests of replicability of prior research findings infeasible. Included in the extended DNA methylation analysis were CpG islands in the promoter regions of *BDNF*, *COMT* and *SLC6A4* as well as CpG regions located in intron 7 of *FKBP5* and exon 6 of *NR3C1.* No statistically significant trends in methylation were observed across the four groups of interest in any of the candidate gene regions under investigation, with the exception of the 3rd CpG island located in the *SLC6A4* promoter region (*SLC6A4*_3). Lack of association could be explained as a lack of effect of childhood trauma or AP on the methylation states of the regions that were investigated, or a lack of statistical power to make relevant inferences. However, it should be noted that only a minor portion of the entire *NR3C1* promoter region could be investigated due to difficulty constructing appropriate primers for the GC-rich downstream sequence.

Alterations in DNA methylation within *SLC6A4* have been well studied. Studies have been largely restricted to investigating DNA methylation in the promoter region, nonetheless it has yielded some interesting results; elevated promoter methylation has been associated with depressive symptoms (Kang et al., 2013b; Phillips et al., 2006; Zhao et al., 2013) and differential methylation has also been associated with treatment response in patients with depression

(Domschke et al., 2014; Okada et al., 2014) as well as anxiety disorders (Roberts et al., 2014). Additionally, *SLC6A4* promoter methylation has also been found to modify the effect traumatic exposure has on risk to develop PTSD (Koenen et al., 2011). In the current study a significant trend in methylation was observed within the 3rd CpG Island of the *SLC6A4* promoter region, whereby methylation significantly increases as level of childhood trauma experienced as well as level of AP increases. However, the statistical significance, although linear, results in minimal percentage increase in DNA methylation across group statuses (as indicated in Figure 6). Previously, elevated methylation in the *SLC6A4* promoter region, including the CpG sites situated in the 3rd CpG island, as a result of early life stress, chronic stress and depressive symptoms, has been found to be associated with a reduction in *SLC6A4* expression in a Caucasian cohort (Duman and Canli, 2015). Therefore, it is possible that the significant linear trend in methylation observed in the 3rd *SLC6A4* CpG island in our study could result in a significant downward trend in gene expression, whereby expression rates decrease as levels of childhood trauma experienced and levels of AP increase. Therefore, elevated exposure to childhood trauma and elevated severity of childhood trauma could illicit elevated DNA methylation in the *SLC6A4* promoter region. The increased methylation clustered at the promoter region could then translate in reduced *SLC6A4* gene expression. The aforementioned should be investigated empirically using RNA extracted from the individuals in the current study in order to measure gene expression. Elevated *SLC6A4* expression has previously been associated with low anxiety phenotype in mice (Jennings et al., 2006; Line et al., 2011) whilst reduced expression has been associated with anxiety-related traits in humans (Lesch et al., 1996), therefore we can speculate that reduced *SLC6A4* expression could lead to increased susceptibility to AP. Additionally, the short-allele of a well-studied insertion-deletion polymorphism within the *SLC6A4* promoter, 5-HTTLPR, has been associated with both increased anxiety and anxiety-related traits (Fergusson et al., 2011; Hemmings et al., 2016; Lesch et al., 1996; Stein et al., 2007a) as well as reduced gene expression (Greenberg et al., 1999; Lesch et al., 1996). A previous study, conducted in the same cohort as currently investigated, found that the long-allele of the 5-HTTLPR polymorphism was associated with reduced AS in the Black sample, while homozygous short-allele homozygotes Coloured males had elevated AS

(Hemmings et al., 2016). Considering that the 5-HTTLPR polymorphism is situated upstream of *SLC6A4*'s transcription start site in the same region as the 3rd CpG island investigated, it is possible that interaction between the genetic (5-HTTLPR) and epigenetic (*SLC6A4* promoter methylation) components are key in influencing *SLC6A4* expression as well as conferring susceptibility towards AS and AP development as a result of childhood trauma exposure.

In order to supplement the information obtained from the 450K BeadChip array, IPA was conducted, aiming to identify biologically significant pathways and disease associations that may play a role in AP development and response to childhood trauma experience, shared across the six comparisons (as summarised in Table 7) conducted. Although the common functional pathways and networks found were predominantly related to basic cellular functions that can be linked to any number of disease phenomena, several noteworthy disease associations were observed. Childhood trauma experience had a significant influence on DNA methylation of genes associated with the neuro-psychological sleep disorders, hypersomnia and dyssomnia (Peter-Derex et al., 2013; Petit et al., 2007). Both of these disorders have previously been linked to anxiety disorders (Dauvilliers et al., 2013; Petit et al., 2007; Staner, 2003). Interestingly, *HLA-DRB1* and *GABRP,* genes enriched in the aforementioned disorders, have also previously been associated with the psychiatric disorders, schizophrenia (Ratta-apha et al., 2015) and bipolar disorder (Craddock et al., 2008; Petryshen et al., 2005), respectively. Furthermore, childhood trauma experience also had a significant influence on DNA methylation of genes associated with immunological disease and metabolic disease, both of which have previously been associated with several psychiatric disorders, including anxiety disorders (Najjar et al., 2013; Salim et al., 2012; Zass et al., 2016). The IPA results, although promising, should be interpreted with caution as unadjusted p-values were used during the analysis. However, these results do indicate the promise of continued investigation of the influence of childhood trauma exposure on DNA methylation, albeit in larger samples, in the South African context.

Limitations

A number of limitations should be taken into account when interpreting the results of the current study. Firstly, the study was limited in terms of sample size relative to other studies in the field. Extensive genome-wide investigations such as the one conducted, typically require much larger sample sizes to make relevant inferences and conclusions. Due to the large amount of multiple comparisons conducted, that results in an increase in false positive detection rates (Hong and Park, 2012), the analysis suffered from inadequate statistical power to detect statistically significant differences and trends. Considering the relatively small sample size and the exploratory nature of the current study, the genome-wide analysis data that was used to select the CpG sites of interest for verification did not account for any multiple correction testing. However, had results been used, that had been corrected for multiple testing, none of the CpG sites of interest would have reached statistical significance. Thus, the findings would be strengthened by replication in larger sample sizes and independent cohorts. Secondly, results were based on a mixed race study sample and although the extent of ethnic stratification on DNA methylation is not yet fully understood, it does influence DNA sequence variation, which in turn has the potential to influence DNA methylation (Huang et al., 2015). However, ethnicity was included as covariate during the analysis and was not found to be associated with DNA methylation in the current study. Thirdly, the current study employed peripheral whole blood to investigate anxiety disorders, which are primarily brain disorders. This introduces two complications to the study: i) the variation that exists in DNA methylation patterns across the diverse blood cell types is not accounted for as whole blood was investigated (Reinius et al., 2012). Recently, a number of statistical tools have been developed to account for this variation (Shiwa et al., 2016) and should be taken into account in the future. ii) the use of peripheral tissue in investigating a brain disorder could be problematic, as studies have shown both concordance (Horvath et al., 2012; Masliah et al., 2013; Walton et al., 2016) and discordance (Farré et al., 2015) between DNA methylation patterns of the brain and blood. However, we expect methylation trends observed in the blood to be a true reflection of trends in the brain as anxiety disorders are not limited to brain illness, but also have large scale systemic implications (Cameron and Nesse, 1988; Salim et al., 2012). Finally, the Illumina HumanMethylation450K Beadchip is not designed to detect hydroxymethylation without targeting it directly using

oxidative bisulfite treatment, therefore any differences that occur due to this mechanism are unaccounted for in the analysis (Lunnon et al., 2016). Currently, no multiplex array has been designed to take both CpG methylation as well as hydroxymethylation changes into account.

Future studies should therefore focus efforts on replicating the observation in the *SLC6A4* promoter region. In order to do this, a larger study sample should be investigated using the same technology as was used in the current study. Although the statistically significant trend in methylation did not seem to translate into a biologically significant difference in methylation, gene expression studies should be conducted, preferably on larger study samples as well, in order to investigate whether the linear trend may influence *SLC6A4* gene expression.

Furthermore, the human brain (structure and functioning) is under large influence of genes from early development well into old age (Peper et al., 2007). Functional imaging genetics and epigenetics are fairly recent approaches that aim to link genetics and epigenetics to behavioural and psychiatric outcomes through brain imaging. Recently, a study combining DNA methylation data acquired from whole blood with brain imaging and lifetime stress evaluation, observed evidence indicative of *in vivo* modulation of brain activity via *COMT* methylation and environmental stress. The study focused its efforts on a notable *Val/Met* polymorphism, rs4680, in *COMT*. A higher degree of DNA methylation was observed in *Val* homozygotes when compared to *Val/Met* heterozygotes and *Met* homozygotes. The higher degree of methylation was inversely correlated with *COMT* gene expression. However, a history of lifetime stress altered the effect and was associated with reduced *COMT* methylation in *Val* homozygotes which correlated with increased prefrontal cortex (PFC) activity during working memory exercises. This was explained as a reduction in cortical efficiency due to stress and the DNA methylation of the *COMT* gene (Ursini et al., 2011). Another study investigated the influence *BDNF* promoter methylation may have on the neural activity of mothers that have experienced intimate partner violence, when exposed to child stimuli of both a stressful and non-stressful nature. Interestingly, maternal *BDNF* methylation was associated with both maternal anxiety as well as exposure to interpersonal violence (Moser et al., 2015). Moreover, maternal *BDNF* methylation was also negatively correlated with hippocampus activity and positively correlated

with activity in both the maternal anterior cingulate and ventromedial prefrontal cortex, brain regions associated with emotion regulation (Moser et al., 2015). Considering the aforementioned studies, Functional imaging epigenetics presents an intriguing avenue of investigation into the effects of childhood trauma and AP on brain structure and functioning and the mechanisms by which DNA methylation could mediate these effects.

To our knowledge, the current study represents one of the first epigenetic studies conducted in the Black and Coloured South African populations. It also represents the first epigenetic study investigating the anxiety disorder endophenotype, AP. The study provides evidence that methylation within the *SLC6A4* promoter increases, linearly, with increased exposure to childhood trauma and levels of AP. No evidence was found that methylation within the other genes under investigation play a role in the development of AP. However the study serves to better characterise and enhance our understanding of the endophenotype and also serves as a useful guideline in how to conduct DNA methylation studies within this cohort.

5. CONCLUSION

The study provides the first evidence that methylation within the promoter region of the *SLC6A4* gene plays a role in the development of AP in Black and Coloured South African adolescents, and a possible role in AS development as well. Methylation within this region may be a biological response to elevated exposure to childhood trauma. Methylation within regions of the *BAI2*, *GALNT9*, *PRKCZ*, *SLC8A2*, *STEAP3*, *BDNF*, *COMT*, *FKBP5* and *NR3C1* were not found to play a role in AP development and were not found to be influenced by childhood trauma experience in the current study either. The study also indicates promise to using whole blood as a biomarker for brain tissue when investigating DNA methylation in the context of anxiety disorders. Additionally, the Illumina 450K BeadChip array provides a useful and comprehensive method of conducting EWAS investigations, however it is crucial to use large study samples when using this technology, in order to make relevant conclusions.

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