Investigating differential expression in PTSD patients versus controls: An RNA-Seq study

by
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ABSTRACT

Post-traumatic stress disorder (PTSD) is a debilitating neuropsychiatric disorder underpinned by complex, multi-factorial interactions including genetic and environmental factors. To date, most genetic studies have focused on specific candidate genes involved in PTSD and therefore lack a holistic view of the disorder. In this study, we aimed to utilise RNA-Seq to investigate molecular mechanisms and possible blood bio-signatures in South African PTSD patients.

Whole blood gene expression levels of South African mixed ancestry ethnicity (Coloured) individuals were compared between PTSD diagnosed (N = 19) and trauma-exposed control (N = 29) individuals. RNA from whole blood from each participant was subjected to RNA-Seq using the Illumina HiSeq 4000 platform at a sequencing depth of 50 million paired-end reads. Differentially expressed genes (p-value < 0.05) were further prioritized based on their involvement in disease phenotype, function, pathways and known gene/protein interactions using the semantic model of disease in BioOntological Relationship Graph (BORG) database. Furthermore, co-expression analysis of the prioritized candidate genes were carried out to investigate co-regulated differentially expressed gene sets between each groups.

A total of 556 differentially expressed genes were identified, of which 196 (21 up- and 175 downregulated) genes were identified as being possibly biologically relevant. Co-expression analysis revealed a network of four highly co-expressed, upregulated genes and a large co-expression network consisting of 36 downregulated genes. The four co-expressed upregulated genes (*RPL6, RPS6, RPS3A* and *EEF1B2*) and six highly connected co-expressed downregulated genes (*DHX9, BCLAF1, THRAP3, EIF4G1, HSPA4* and *MCL1*) were identified as potentially relevant gene candidates contributing to the pathology of PTSD.

In conclusion, we were able to identify putative blood transcriptomic response in PTSD patients' vs trauma-exposed controls. Additionally, a set of differentially expressed genes, possibly associated with molecular functions/mechanisms of PTSD were determined. These preliminary findings provide novel insight in underlying genetic expression of PTSD in South African population. Future transcriptomic studies using larger sample size will be instrumental in validating our findings, and should include miRNA profiling to identify a more robust signature of potential blood based biomarkers.

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OPSOMMING

Post-traumatiese stresversteuring (PTSV) is 'n neuropsigiatriese siekte wat bestaan uit komplekse, multi-faktoriaal interaksies. To top hede het meeste genetiese studies slegs gefokus op spesifieke kandidaat gene betrokke by PTSV. Hierdie kandidaat studies het dus nie 'n holistiese siening wat kan verkry word deur 'n hele-transkriptoom RNS-Sequencing (RNS-Seq) benadering nie. In hierdie voorlopige studie beoog ons om RNS-Seq aan te wend om molekulêre meganismes en moontlike bloed biomerkers in Suid-Afrikaanse PTSV patiente te ondersoek.

In hierdie kontrole studie vergelyk vroulike, kleurling (gemengde afkoms) individue wat gediagnoseer is met PTSV (N = 19) met ‘n trauma blootgestelde kontrole (N = 29) groep. RNS was geisoleer vanaf vol bloed en gestuur vir RNS-Seq met behulp van die Illumina HiSeq 4000 platform op 'n opeenvolging diepte van 50 miljoen lees pare. Bioinformatika ontledings was toe uitgevoer, gevolg deur stroomaf mede-uitdrukking analise om mede-gereguleerde differensieel uitgedruk gene stelle tussen groepe te ondersoek.

'n Totaal van 556 differensieel uitgedruk gene was geïdentifiseer waarvan 196 (21 opreguleer en 175 onderreguleer) gene biologies relevant was gebaseer is op 'n ontologie gedryfde prioriteits benadering. Mede-uitdrukking analise het daarna 'n netwerk van vier hoogs mede-uitgedrukkings gene (opreguleer) en 'n groot mede-uitdrukking netwerk van 36 gene (onderreguleer) geïdentifiseer. Die vier mede-uitgespreek gene (*RPL6, RPS6, RPS3A* en *EEF1B2*) (opreguleer) en ses hoogs verbind mede-uitgespreek gene (*DHX9, BCLAF1, THRAP3, EIF4G1, HSPA4* en *MCL1*) (onderreguleer) was geïdentifiseer as potensieel, relevante skakels wat bydra tot die patologie van PTSV.

Hierdie hipotese-genererende studie dien as ondersteunende bewys dat 'n bloed transkriptomise reaksie betrokke by PTSV. Hierbenewens het die studie gene geidentifiseer wat moontlik betrokke is by die molekulêre onderbou van hierdie siekte. Toekomstige studies word egter aanbeveel om hierdie bevindinge te ondersteun en om miRNA profilering te gebruik vir die identifisering van meer robuuste, bloed gebaseer biomerkers vir PTSV.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
μg	microgram
μl	microliter
˚C	degrees Celsius
3'	three prime
3' UTR	three prime untranslated region
5'	five prime
A	adenine
ACTH	adrenocorticotrophic hormone
AVP	arginine-vasopressin
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BLA	basolateral nucleus
BORG	BioOntological Relationship Graph
bp	base pair
C	cytosine
Ca2+	calcium
CAF	Central Analytical Facilities
CAPS	Clinician Administered Posttraumatic Stress Disorder Scale
CeA	central nucleus of the amygdala
CR	conditioned response
CRH	corticotrophin-releasing hormone
CS	conditioned stimulus
CSDS	chronic social defeat stress
CTQ	Childhood Trauma Questionnaire
CVD	cardiovascular disorders
dACC	dorsal anterior cingulate cortex
DEG	differentially expressed gene
dmPFC	dorsomedial prefrontal cortex

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DNA	Deoxyribo Nucleic Acid
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, version V
DZ	dizygotic
EMBL	European Molecular Biology Laboratory
FKBP5	FK506 binding protein 5 gene
fMRI	functional MRI
G	guanine
g	gram
GABA	gamma-aminobutyric acid
G x E	gene-environment
GC	glucocorticoid
GO	Gene Ontology
GR	glucocorticoid receptor gene
GTEx	Genotype-Tissue Expression portal
GTF	gene transfer format
GWAS	genome-wide association studies
HISAT2	hierarchical indexing for spliced alignment of transcripts
HPA	hypothalamic–pituitary–adrenal
HPO	Human Phenotype Ontology
Hsa	*Homo sapiens*
KEGG	Kyoto Encyclopedia of Genes and Genomes
LEC	Life Events Checklist
MDD	major depressive disorder
MetS	metabolic syndrome
min	minutes
miRNA	micro RNA
ml	millilitres
MPO	Mammalian Phenotype Ontology
mPFC	medial prefrontal cortex
MR	mutual rank
MRC	Medical Research Council
MRI	magnetic resonance imaging
MZ	monozygotic

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NCBI	National Centre for Bioinformatics
NGS	Next-generation sequencing
NMDA	N-methyl-D-aspartate
NPD	neuropsychiatric disorder
nt	nucleotide
OMIM	Online Mendelian Inheritance in Man
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	paired-end
POMC	proopiomelanocortin
PTSD	post-traumatic stress disorder
PVN	parvocellular neurons in the paraventricular nucleus
PW	Pathway Ontology
rRNA	ribosomal RNA
RNA	ribonucleic acid
RNA-Seq	RNA sequencing
RIN	RNA integrity numbers
RORA	retinoid-related orphan receptor alpha gene
ROS	reactive oxygen species
SANBI	South African National Bioinformatics Institute
SBS	sequencing by synthesis
SNP	single nucleotide polymorphism
SPS	single prolonged stress
T	thymine
TE	trauma-exposed
tRNA	total ribonucleic acid
US	unconditioned stimulus
vmPFC	ventromedial prefrontal cortex

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CHAPTER 1 : INTRODUCTION

1.1 Background of PTSD

Post-traumatic stress disorder (PTSD) is a debilitating neuropsychiatric disorder, triggered by life-threatening, traumatic or stressful events (American Psychiatric Association, 2013), significantly impairing an individual's functioning and overall quality of life (Mendlowicz & Stein, 2000). Moreover, this stress-related disorder poses an immense economic and health burden on society (Atwoli *et al*., 2013). Classified as a trauma- and stressor-related disorder in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V), PTSD is characterised by four major behavioural symptom clusters, including (i) re-experiencing, (ii) avoidance, (iii) hyperarousal and (iv) overall negative alterations in cognition and mood (American Psychiatric Association, 2013).

Both clinical and demographic factors play a role in the increased risk for PTSD, with females at an overall two-fold higher risk than males of developing PTSD following trauma exposure (Breslau, 2009). The reason for these differences remains unclear, warranting further research focusing on PTSD, and in women in particular. Other risk factors for PTSD include a lack of social support structure, childhood abuse or neglect and the severity and duration of the trauma. Accounting for these factors may allow for early diagnosis of PTSD and possible preventive strategies to reduce the symptoms associated with this debilitating disorder (Broekman, Olff & Boer, 2007).

South Africa has one of the highest prevalence rates for trauma exposure, estimated at 73.8% according to the South African Stress and Health Study (Atwoli *et al*., 2013). This may be due to the historical, cultural and political factors faced in South Africa's past as well as the high levels of criminal violence still present today. Countries such as the USA, Brazil, Peru and Australia reported similar prevalence rates of trauma exposure to that of South Africa (above 70%) whilst countries such as China, Spain, Romania and Bulgaria reported much lower prevalence rates (less than 55%) for exposure to any traumatic event (Benjet *et al*., 2016).

Interestingly, approximately 2.3% of South African individuals who are exposed to a traumatic event will develop PTSD (Herman *et al*., 2009). This estimate is significantly lower than the lifetime prevalence rates in Europe (7.4%) (de Vries & Olff, 2009) and in North America (6.8%) (Kessler *et al*., 2005). This cross-national variation could in part be explained by the higher instances of traumatic event exposure within South Africa (Herman *et al*., 2009). These exposures could make it difficult to fulfil the avoidance criteria of the DSM-V, possibly leading to an underrepresentation of PTSD diagnosis in the country (Atwoli *et al*., 2013).

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Not all individuals who have undergone a traumatic event will develop PTSD (Monroe, Simons & Thase, 1991; Costello *et al*., 2002), suggesting that trauma exposure alone does not explain the complete aetiology of the disorder. Other risk factors, such as genetics, have been found to increase vulnerability to developing this stress-related disorder. Interest in the genetic underpinnings of PTSD has grown, leading to research exploring the molecular risk and developmental factors involved in this debilitating disorder (Glatt *et al*. 2013; Breen *et al*. 2015; Tylee *et al*. 2015). However, due to the genetic complexity of PTSD, identifying specific genes that significantly contribute to disease development has been a challenge.

To investigate the genetic mechanisms involved in PTSD it is essential to review the physiological stress responses involved in disease pathophysiology. This will facilitate the identification of the molecular underpinnings of PTSD.

1.2 Physiological systems involved in PTSD

Acute stress leads to the activation of the "fight-or-flight" response which in turn activates the neurocircuitry of the fear system, the hypothalamic-pituitary-adrenal (HPA) axis, the locus coeruleus and the noradrenergic systems (Charney *et al*., 1995). From an evolutionary standpoint, the "fight-or-flight" response assists our identification of danger and allows us to avoid similar threats in future. However, this adaptive response has similarly been implicated in fear conditioning, which plays an integral role in PTSD pathophysiology (Amstadter, Nugent & Koenen, 2009).

Fear conditioning is a form of classical conditioning where associative learning plays a pivotal role in the maintenance of fear (Keane, Zimering & Caddell, 1985). Classical conditioning is a process whereby a non-threatening stimulus, termed the conditioned stimulus (CS) is temporarily paired with a fear stimulus termed the unconditioned stimulus (US). After this temporary pairing the CS will ultimately provoke a fear response similar to that of the US termed the conditioned response (CR) (Foa, Steketee & Rothbaum, 1989; Grillon *et al*., 1998). In the case of PTSD, the trauma exposure serves as the US whilst smell, sight, sounds and other environmental stimuli experienced during the traumatic event serves as the CS eliciting a CR to seemingly non-threatening stimuli (Skelton *et al*., 2012).

In the following section, neurobiological pathways implicating the fear-conditioning model and its association with PTSD will be reviewed in greater detail.

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1.2.1 The Hypothalamic-Pituitary-Adrenal Axis in PTSD

The hypothalamic-pituitary-adrenal (HPA) axis, which is an important regulator of stress response, interacts with the immune system to maintain biological homeostasis in humans and mammals (Mehta & Binder, 2012). During a typical stress response, the HPA axis reacts to acute stress by activating a cascade of signalling, mobilised by the sympathetic nervous system for an acute "fight-or-flight" response (Figure 1.1) (Griffiths & Hunter, 2014). The first process in the signalling cascade is stress-induced activation of the parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus, stimulating the release of the neuropeptides, corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP), into the pituitary portal. This release of CRH and AVP in response to stress promotes the production of proopiomelanocortin (POMC) in the anterior pituitary, which synthesises and releases adrenocorticotrophic hormone (ACTH) into systemic circulation (Aguilera, 2012). The ACTH in turn acts on the adrenal cortex to produce and release cortisol, a glucocorticoid (GC) hormone which is primarily responsible for the stress response and exerts its action on the immune response, metabolism and brain function (Zoladz & Diamond, 2013). Cortisol further functions as a regulator of the HPA axis by utilizing a negative feedback mechanism to adapt and recover from stress by restore biological homeostasis (Figure 1.1)(Yehuda *et al*., 2006). In this negative feedback mechanism an excess of cortisol binds to glucocorticoid receptors (GRs) within the hypothalamus and pituitary, suppressing the release of CRH and ACTH, thereby returning the HPA axis to baseline activity and allowing for the restoration of biological homeostasis and the adaption and recovery from a stress response (Griffiths & Hunter, 2014).

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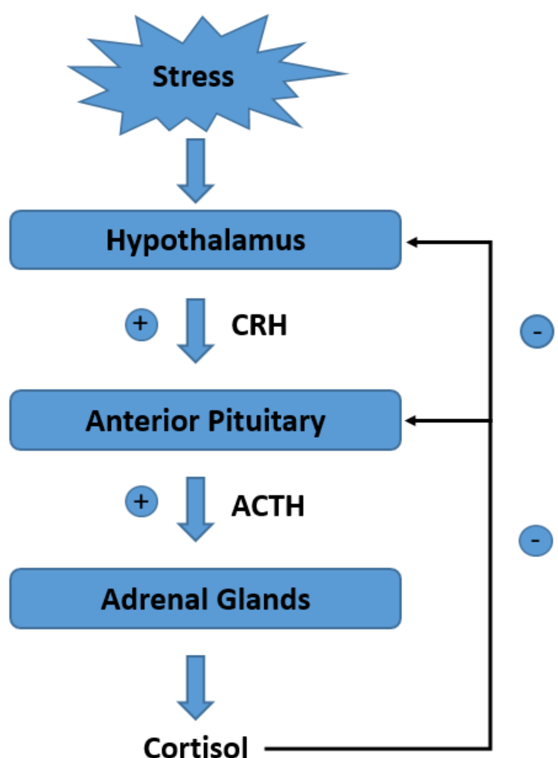

Figure 1.1: Schematic representation of the effect of stress on the hypothalamic-pituitary-adrenal (HPA) axis. Stress activates a cascade of signalling, resulting in an acute "fight-or-flight" response. This induces the activation of neurons located in the paraventricular nucleus (PVN) of the hypothalamus, which stimulates the release of corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP) into the anterior pituitary. This promotes the production of proopiomelanocortin (POMC) synthesises and releases adrenocorticotrophic hormone (ACTH) into systemic circulation. ACTH then acts on the adrenal cortex to produce and release cortisol. Cortisol furthermore regulates the HPA axis by supressing the release of CRH and ACTH restoring biological homeostasis after a stress response (Adapted from Griffiths & Hunter, 2014).

Studies investigating components of the HPA axis in PTSD have led to conflicting results. Some studies have indicated a decrease in urinary cortisol levels (collected over a period of 24 hours) within PTSD patients (Mason *et al.*, 1986; Yehuda *et al.*, 1990) whilst others (Mason *et al.*, 2002) detected no differences. Similarly, a study investigating blood plasma cortisol levels (over a period of 24 hours) reported decreased cortisol levels in combat veterans with PTSD compared to control individuals (Yehuda *et al.*, 1994, 1996). In contrast a study by Goenjian *et al.*, (2003) reported no differences in plasma cortisol levels in an adolescent group with PTSD symptoms compared to controls (Goenjian *et al.*, 2003). Several other studies have also reported decreased cortisol levels (Yehuda *et al.*, 1990, 1996, 2006; Thaller *et al.*, 1999; Bremner, Elzinga & Schmahl, 2007) and increased levels of CRH in PTSD patients (Bremner *et al.*, 1997; Baker *et al.*, 1999; Bremner, Elzinga & Schmahl, 2007), suggesting that an enhanced negative feedback of the HPA axis could be involved in PTSD (Griffin, Resick & Yehuda, 2005; Yehuda *et al.*, 2006). Inconsistencies in cortisol levels

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(Mason *et al*., 1986; Yehuda *et al*., 1990, 1996, 2006; Thaller *et al*., 1999; Mason *et al*., 2002; Goenjian *et al*., 2003; Bremner, Elzinga & Schmahl, 2007) may in part be due to, the differences in index trauma experienced, age or even due to a genetic vulnerability (Pervanidou & Chrousos, 2010).

1.2.2 The Neurobiological Pathways of PTSD

Post-traumatic stress disorder has been associated with certain neurobiological abnormalities leading to the inability of the brain to adequately extinguish fear (Bremner *et al*., 1996). However, some debate remains as to whether these abnormalities are a cause or a determining factor of the disorder. Brain regions commonly investigated in PTSD include the hippocampus, amygdala, insular cortex and regions of the medial prefrontal cortex (mPFC), including the (vmPFC) and the dorsal anterior cingulate cortex (dACC) (Quirk & Mueller, 2008) (Figure 1.2).

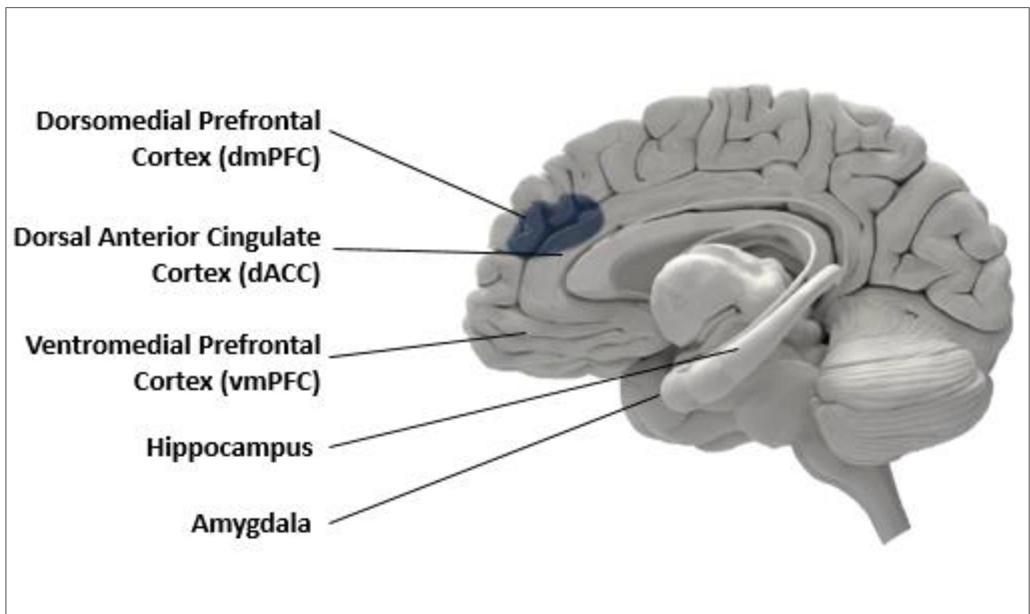

Figure 1.2: Brain regions frequently investigated in PTSD. A schematic representation of the midsagittal plane of the brain and regions implicated in PTSD (Adapted from Liberzon & Sripada, 2007).

1.2.2.1 Hippocampus

Post-traumatic stress disorder is associated with memory deficits, especially in declarative memory (memories that can be consciously be recalled) which forms part of the long-term memory in humans (Francati, Vermetten & Bremner, 2007). The brain structure known as the hippocampus is essential for merging information from short-term memory to long-term memory in a process known as memory consolidation. The hippocampus thus plays a critical role in the pathogenesis of PTSD and

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the symptoms of often re-experiencing a traumatic event (Bremner *et al*., 2003; Pervanidou & Chrousos, 2010).

To date, imaging studies in PTSD have focused mostly on the volumetric changes of the hippocampus by use of magnetic resonance imaging (MRI) (structural imaging). In a study by Bremner, Elzinga & Schmahl, (2007), a decrease in hippocampal volume was reported in a group of Vietnam veterans with PTSD and in patients suffering from chronic PTSD (Bremner, Elzinga & Schmahl, 2007). Several other brain imaging studies reported similar findings of reduced hippocampal volume and function in PTSD patients compared to trauma-exposed controls (Liberzon & Martis, 2006; Wang *et al*., 2010) (Table 1.1). However, whether these hippocampal volume changes are due to extreme trauma or a risk factor of PTSD remains unclear. Furthermore, decreased levels of N-acetyl aspirate in the hippocampus has also been observed in MRI studies (Rauch, Shin & Phelps, 2006) whilst functional magnetic resonance imaging (fMRI) studies revealed deficits in verbal declarative memory task in PTSD patients, a process mediated by the hippocampus (Francati, Vermetten & Bremner, 2007).

1.2.2.2 Amygdala

The amygdala forms part of the limbic system located within the temporal lobe of the brain (Davis, 1992) (Figure 1.2). This brain region functions as a centre for decision-making, memory processing/learning, emotional reactions and in HPA axis activation. In terms of PTSD the amygdala plays a central role in behavioural responses such as fear response, threat detection and especially in fear conditioning (Davis, 1992) (Table 1.1).

The amygdala consists of several nuclei, with the central nucleus of the amygdala (CeA) and the basolateral nucleus (BLA) playing a central role in fear conditioning (Jovanovic & Ressler, 2010). The BLA is responsible for the acquisition of fear by associating a CS to that of an US and in turn projects this information to the CeA which is responsible for regulating particular aspects of the fear response (LeDoux, 1992). These findings have been observed in animal studies where lesions in the CeA reduced the fear condition responses of rodents by eliminating the freeze response (LeDoux, 1992) and the fear-potentiated startle response (Davis, Gendelman & Tischler, 1982).

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Table 1.1: Functional and structural neuroimaging studies of brain regions implicated in PTSD relative to trauma-exposed controls. (Adapted from Thakur *et al*., 2015)

	Functional imaging			**Structural imaging**
Brain regions	**General function**	**Activity in PTSD subjects**	**Correlation with PTSD severity**	**Overall volume**
Hippocampus	Short- and long-term memory	Varied (Bremner *et al*., 2003; Shin & Handwerger, 2009; Sripada *et al*., 2013; Steiger *et al*., 2015)	Negative (Gilbertson *et al*., 2002)	Decreased (Liberzon & Martis, 2006; Wang *et al*., 2010)
Amygdala	Threat detection, processing of fear	Increased (Liberzon *et al*., 1999; Etkin & Wager, 2007; Linnman *et al*., 2011)	Positive (Shin *et al*., 2004, 2005)	Varied (Etkin & Wager, 2007)
vmPFC	Goal-directed decisions	Decreased (Shin *et al*., 2004; Felmingham, Williams & Kemp, 2009; Gold *et al*., 2011)	Negative (Shin *et al*., 2004; Milad *et al*., 2009)	Decreased (Kasai, Yamasue, Gilbertson & Shenton, 2008; Karl & Werner, 2010; Sekiguchi *et al*., 2013)
dACC	Regulating cognitive control, fear appraisal and expression	Increased (Milad *et al*., 2009; Hayes *et al*., 2011; Shvil *et al*., 2014)	Positive (Milad *et al*., 2009; Fonzo *et al*., 2010)	Decreased (Kitayama, Quinn & Bremner, 2006; Kasai, Yamasue, Gilbertson & Shenton, 2008; Karl & Werner, 2010; Sekiguchi *et al*., 2013)
Insular cortex	Monitors interpersonal experiences	Increased (Simmons *et al*., 2008; Strigo *et al*., 2010)	Positive (Simmons *et al*., 2008)	Decreased (Simmons *et al*., 2008)

vmPFC - ventromedial prefrontal cortex ; dACC - dorsal anterior cingulate cortex

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1.2.2.3 Prefrontal cortex and anterior cingulate cortex

Another brain region critical in the regulation of the fear conditioning response is the medial prefrontal cortex (mPFC). Here reciprocal connections between the amygdala and the mPFC, a brain region playing a major role in fear extinction, are critical in the inhibition of the stress response and fear reactions (Milad & Quirk, 2002; Vidal-Gonzalez *et al*., 2006; Peters, Kalivas & Quirk, 2009).

The prefrontal cortex can be subdivided into regions including the orbitofrontal, medial prefrontal cortex and the anterior cingulate cortex (ACC). The ACC consists of ventromedial and dorsolateral components which are responsible for regulating the expression and inhibition of fear in different ways. Brain imaging studies showed a decrease in activity (Shin *et al*., 2004; Felmingham, Williams & Kemp, 2009; Gold *et al*., 2011) and volume (Kasai, Yamasue, Gilbertson, Shenton, *et al*., 2008) of the vmPFC in PTSD patients including decreased volumes of the anterior cingulate cortex (Rauch *et al*., 2003; Kitayama, Quinn & Bremner, 2006; Kasai, Yamasue, Gilbertson, Shenton, *et al*., 2008) and medial frontal gyrus (Carrion *et al*., 2001; Fennema-Notestine *et al*., 2002; Rauch *et al*., 2003; Yamasue *et al*., 2003; Woodward *et al*., 2006). Additionally, studies by Bremner *et al*., (1999) and Britton *et al*., (2005) made use of functional imaging studies identifying decreased activation of the mPFC in PTSD individuals in response to stimuli such as combat pictures and sounds (Bremner *et al*., 1999; Britton *et al*., 2005) (Table1.1).

1.2.2.4 Insular cortex

The insular cortex, which forms part of the cerebral cortex, is involved in consciousness (monitoring internal body states). This includes our perception, motor control, self-awareness, cognitive functioning and interpersonal experience. In terms of PTSD an overall increased activity has been previously observed in the insular cortex (Simmons *et al*., 2008; Strigo *et al*., 2010) with structural imaging studies identifying an overall decrease in volume (Simmons *et al*., 2008) (Table 1.1).

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1.3 The genetic aetiology of PTSD

Various family and twin studies indicate that PTSD is a heritable disorder (Skre *et al*., 1993; True *et al*., 1993; Xian *et al*., 2000; Stein *et al*., 2002; Kasai, Yamasue, Gilbertson, Shenton, *et al*., 2008; Amstadter *et al*., 2012), suggesting that a genetic predisposition exists in the development of this debilitating disorder after the occurrence of a traumatic event.

1.3.1 Heritability of PTSD: Family and Twin Studies

Family studies by Sack, Clarke & Seeley, (1995) and Yehuda, Halligan & Grossman, (2001) indicated that the prevalence of PTSD is higher in relatives of PTSD patients compared to relatives of trauma-exposed controls, suggesting that the vulnerability to develop PTSD runs within families (Sack, Clarke & Seeley, 1995; Yehuda, Halligan & Grossman, 2001). However, it could be argued that biological relatives share more environmental exposures and are therefore more vulnerable to developing PTSD. Twin studies allow for the separation of environmental and genetic factors involved in disease development. In PTSD, these studies have estimated that 30% to 40% of this heritability is due to genetic factors. However, twin studies do not indicate which genes lead to an increased risk for PTSD (Koenen, 2007; Kasai, Yamasue, Gilbertson, Shenton, *et al*., 2008; Afifi *et al*., 2010). Therefore, molecular studies are crucial in the identification of genes involved in the genetic aetiology.

1.3.2 Candidate Gene Studies in PTSD

By identifying potential genes involved in PTSD, it is possible to improve our understanding of factors involved in the development, maintenance and treatment of PTSD (Amstadter, Nugent & Koenen, 2009). To date, most molecular genetic research in PTSD focused on candidate gene studies. Candidate gene studies identify risk variants associated with disease. These genetic risk variants are referred to as polymorphisms, which include single nucleotide polymorphisms (SNPs) and variable number tandem repeats (VNTRs). Candidate gene studies rely on prior knowledge of the biological pathways involved in the particular disease informing the selection of potential candidate gene that may be involved in PTSD (Amstadter, Nugent & Koenen, 2009). Table 1.2 outlines several published candidate genes investigated in PTSD.

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Table 1.2: Summary of published candidate genes studies investigated in PTSD. (Adapted from Cornelis *et al.*, 2010 and Voisey *et al.*, 2013)

				PTSD Cases			**Controls**			**Co-morbidities accounted for**		
Reference	**Gene**	**Polymorphism**	**Finding**	**Number**	**Sex (% male)**	**Age, mean (SD)**	**Number**	**Sex (% male)**	**Age, mean (SD)**	**Cases**	**Controls**	**Population**
(Comings *et al.*, 1991)	*ANKK1*	rs1800497	T associated	35	All male	N/S	314	All male	N/S	Yes	Yes	USA, Eur
(Comings, Muhleman & Gysin, 1996)	*ANKK1*	rs1800497	T associated	37	All male	~44	19	All male	~44	Yes	Yes	USA, Eur
(Gelernter, Kranzler & Satel, 1999)	*ANKK1*	rs1800497	No association	52	All male	45 (4)	87	All male	N/S	Yes	Yes	USA, Eur
	DRD2	rs1079597	No association									
		rs1800498	No association									
(Young *et al.*, 2002)	*ANKK1*	rs1800497	T associated	91	All male	52 (1)	51	35%	39 (2)	Yes	N/S	Aus, Eur
(Voisey *et al.*, 2008)	*ANKK1*	rs1800497	No association	127	All male	N/S	228	N/S	N/S	No	N/S	Aus, Eur
	DRD2	rs6277	C associated									
		rs1799732	No association									
(Nelson *et al.*, 2014)	*DRD2*	rs12364283	G associated	651	47%	~36 (~9)	1098	65%	~36 (~9)	Yes	Yes	Aus, Mixed
(Hemmings *et al.*, 2013)	*ANKK1/ BDNF*	rs1800497/ rs6265	T/Val associated	150	31%	23-42	N/A	N/A	N/A	Yes	N/A	South African, non-Eur
	SLC6A4	5'-VNTR	No association									
(Dragan & Oniszczenko, 2009)	*DRD4*	VNTR exon3	L-allele associated	24	~47%	~36	83	~47%	~36	N/S	N/S	Polish
	SLC6A3	rs28363170	No association	70	N/S	N/S	130	N/S	N/S	Yes	Yes	
(Valente, Vallada, Cordeiro, Miguita, *et al.*, 2011)	*SLC6A3*	rs28363170	9 Repeat associated	65	33%	38 (`8.7)	34	17.60%	44 (~13.8)	Yes	Yes	Brazilian, Mixed
	BDNF	rs6265	No association									
	SLC6A4	5'-VNTR	No association									
(Segman *et al.*, 2002)	*SLC6A3*	rs28363170	9 Repeat associated	102	56%	40 (12)	104	47%	34 (10)	No	No	Israel
(Drury *et al.*, 2009)	*SLC6A3*	rs28363171	9 Repeat associated	88	59%	Range (3-6)	88	59%	3-6 (range)	No	No	USA, AA & other
(Chang *et al.*, 2012)	*SLC6A3*	rs28363172	9 Repeat associated	62	35%	61	258	43%	52	Yes	Yes	USA, AA & other
(Drury *et al.*, 2013)	*SLC6A3*	rs28363170/rs27 072	Haplotype associated	66	N/S	Range (3-6)	77	N/S	3-6 (range)	No	No	USA, AA & other

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(Lee *et al*., 2005)	*SLC6A4*	5'-VNTR	S-allele associated	100	43%	35 (10)	197	39%	35 (11)	No	No	Korean
(Kilpatrick *et al*., 2007)	*SLC6A4*	5'-VNTR/rs25531	S+-haplotype associated	19	32%	Adults	570	37%	Adults	Yes	Yes	USA, Mixed
(Grabe *et al*., 2009)	*SLC6A4*	5'-VNTR/rs25531	L/A haplotype associated	67	36%	58 (17)	1596 (TE) 1382 (NTE)	51%, 46%	58 (16), 50 (13)	Yes	Yes	German, Eur
(Koenen *et al*., 2009)	*SLC6A4*	5'-VNTR	S-allele associated	19	32%	Adults	571	36%	Adults	Yes	Yes	USA, Mixed
(Kolassa, Ertl, *et al*., 2010)	*SLC6A4*	5'-VNTR	S-allele associated	331	~53%	~35	77	~53%	~35	No	No	Rwandan
(Sayin *et al*., 2010)	*SLC6A4*	5'-VNTR Intron2 VNTR	S-allele associated 12 rept associated	29	38%	N/S	48	75%	N/S	Yes	Yes	Turkey, Eur
(Thakur, Joober & Brunet, 2009)	*SLC6A4*	5'-VNTR	L/L associated	24	~46%	~30	17	~46%	~30	N/S	N/S	USA, Eur
(Xie *et al*., 2009)	*SLC6A4*	5'-VNTR/rs25531	S+-haplotype associated	229	42%	39 (10)	1023	54%	39 (11)	Yes	Yes	USA, Eur & AA
(Walsh *et al*., 2014)	*SLC6A4*	5'-VNTR/rs25531	S+-haplotype associated	205	N/S	N/S	477	N/S	N/S	No	No	USA, AA
(Goenjian *et al*., 2012)	*SLC6A4*	5'-VNTR	S-allele associated	70	N/S	N/S	130	N/S	N/S	Yes	Yes	Armenian, Eur
	TPH1	rs2108977	T associated									
	TPH2	rs11178997	T associated									
(Mellman *et al*., 2009)	*SLC6A4*	5'-VNTR/rs25531	No association	55	24%	40 (16)	63	45%	40 (17)	Yes	Yes	USA, Eur & AA
	HTR2A	rs6311	G associated									
(Lee *et al*., 2007)	*HTR2A*	rs6311	GG associated in females	107	42%	34 (10)	161	32%	32 (10)	No	No	Korean
(Uddin *et al*., 2013)	*ADCYAP1R1*	rs2267735	C associated, females	23	All female	Adults	378	All female	Adults	Yes	Yes	USA, AA & other
(Wang *et al*., 2013)	*ADCYAP1R1*	rs2267735	C associated, females	146	44%	45 (11.6)	174	N/S	45 (11.6)	Yes	Yes	Chinese, Mixed
(Lee *et al*., 2006)	*BDNF*	rs6265	No association	107	42%	34 (10)	161	32%	32 (10)	Yes	Yes	Korean
(Zhang *et al*., 2006)	*BDNF*	rs6265 G712A C270T	No association	96	76%	44 (7)	250	41%	38 (20)	N/S	No	USA, Eur
(Pivac *et al*., 2012)	*BDNF*	rs6265	A associated	206	All male	42 (~7.1)	370	All male	42 (~7.1)	N/S	N/S	Croatian

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(Felmingham *et al.*, 2013)	*BDNF*	rs6265	A associated	55	N/S	N/S	N/S	N/S	N/S	Yes	Yes	Aus, Eur
(Freeman *et al.*, 2005)	*APOE*	rs7412 rs429358	T/T haplotype associated	54	All male	53 (6)	N/A	N/A	N/A	Yes	N/s	USA, Eur
(Kim *et al.*, 2013)	*APOE*	rs7412 rs429358	T/T haplotype associated	128	All male	Adults	128	All male	Adults	Yes	Yes	Korean
(Lyons *et al.*, 2013)	*APOE*	rs7412 rs429358	C/C haplotype associated	39	All male	Adults	131	All male	Adults	Yes	Yes	USA, Eur & other
(Lu *et al.*, 2008)	*CNR1*	rs806369	Haplotypes associated	25	24%	N/S	291	52%	N/S	Yes	Yes	USA, Eur
		rs1049353	A associated									
		rs806377	No association									
		rs6454674	No association									
(Lu *et al.*, 2008)	*CNR1*	rs806369	No association	17	29%	N/S	292	67%	N/S	Yes	Yes	Finland, Eur
		rs1049353	No association									
		rs806377	No association									
		rs6454674	No association									
		rs1049353	No association									
(Binder *et al.*, 2008)	*FKBP5*	rs9296158	A associated	762	~43%	~41 (14)	N/S	N/S	N/S	Yes	N/S	USA, AA & other
		rs3800373	C associated									
		rs1360780	T associated									
		rs9470080	T associated									
		rs992105	No association									
		rs737054	No association									
		rs1334894	No association									
		rs4713916	No association									
(Xie *et al.*, 2010)	*FKBP5*	rs9296158	No association	343	~54%	~39 (11)	2084	~54%	~39 (11)	Yes	Yes	USA, Eur & AA
		rs3800373	No association									
		rs1360780	No association									
		rs9470080	T associated									
	COMT	rs4680	A associated									
	CHRNA5	rs16969968	A associated									
(Valente, Vallada, Cordeiro, Bressan, *et al.*, 2011)	*COMT*	rs4680	A associated	65	33%	38 (~8.7)	34	17.60%	44 (~13.8)	Yes	Yes	Brazil mixed
(Kolassa, Kolassa, *et al.*, 2010)	*COMT*	rs4680	No association	340	~53%	~35	84	~53%	~35	No	No	Rwandan

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(Schulz-Heik *et al.*, 2011)	*COMT*	rs4680	A associated	51	94%	49	48	92%	47	Yes	Yes	USA, Eur & other
(de Quervain *et al.*, 2012)	*PRKCA*	rs4790904	A associated	134	N/S	34 (median)	213	N/S	34 (median)	Yes	Yes	Rwandan
(Liu *et al.*, 2013)	*PRKCA*	rs4790904	G associated	391	~77%	38 (median)	570	~77%	38 (median)	Yes	Yes	USA, Eur & AA
(Logue, Solovieff, *et al.*, 2013)	*ANK3*	rs9804190 rs1049862 rs28932171 rs11599164 rs17208576	C associated T associated T associated G associated G associated	295	N/S	52	196	N/S	52	Yes	Yes	USA, Eur
(Duan *et al.*, 2014)	*CAT*	rs208679 rs10836233 rs2300182 rs769217 rs7104301 rs7949972	No association No association No association No association No association No association	173	60%	36 (6.9)	287	61%	35 (median)	Yes	No	Han Chinese
(White *et al.*, 2013)	*CRHR1*	rs12938031 rs479288 rs173365 rs17689966 rs242924 rs2664008 rs171441 rs16940686 rs242939 rs242936 rs7209436 rs11040	A associated C associated No association No association No association No association No association No association No association No association No association No association	564	36%	Adults	NA	NA	NA	No	NA	USA, Eur
(Mustapi *et al.*, 2007)	*DBH*	rs1611115	No association	133	All male	40 (7)	34	All male	38 (4)	No	No	Croatian
(Nelson *et al.*, 2009)	*GABRA2*	rs279836 rs279826 rs279871 rs279858	T associated A associated A associated No association	46	N/S	N/S	213	N/S	N/S	Yes	Yes	N/S
(Morris *et al.*, 2012)	*KPNA3*	rs2273816	No association	121	All male	52 (6.2)	237	59%	36.8 (12.8)	Yes	No	Aus, Eur
(Lawford *et al.*, 2013)	*NOS1AP*	rs386231	A associated	122	All male	52 (6.2)	237	59%	36.8 (12.8)	Yes	No	Aus, Eur

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(Lappalainen *et al*., 2002)	*NPY*	rs16139	No association	77	All male	N/S	202	All male	N/S	Yes	Yes	USA, Eur
(Bachmann *et al*., 2005)	*NR3C1*	rs6189	No association	118	All male	56 (4)	42	All male	61 (7)	No	No	Aus, Eur
		rs6190	No association									
		rs56149945	No association									
(Amstadter *et al*., 2009)	*RGS2*	rs4606	C associated	273	35%	Adults	334	35%	Adults	Yes	N/S	USA, Eur & other
(Cao *et al*., 2013)	*STMN1*	rs182455	C associated, females	146	28%	Adults	174	30%	Adults	No	No	Chinese, Mixed
(Wilker *et al*., 2013)	*WWC1*	rs10038727	G associated	212	N/S	Adults	579	N/S	Adults	No	No	Rwandan and Ugandan
		rs4576167	G associated									

N/S – Not Stated; N/A – Not Applicable; Aus – Australian; Eur – European; USA – American; *ADCYAP1R1* - ADCYAP Receptor Type I ; *ANK3* - Ankyrin 3; *ANKK1* - Ankyrin repeat and kinase domain containing I; *APOE* - Apolipoprotein E; *BDNF* - Brain Derived Neurotrophic Factor; *CAT* - Catalase; *CHRNA5* - Cholinergic Receptor Nicotinic Alpha 5 Subunit; *CNR1* - Cannabinoid Receptor 1; *COMT* - Catechol-O-Methyltransferase; *CRHR1* - Corticotropin Releasing Hormone Receptor 1; *DBH* - Dopamine Beta-Hydroxylase; *DRD2* - Dopamine Receptor D2; *DRD4* - Dopamine Receptor D4; *FKBP5* - FK506 Binding Protein 5; *GABRA2* - Gamma-Aminobutyric Acid Type A Receptor Alpha2 Subunit; *HTR2A* - 5-Hydroxytryptamine Receptor 2A; *KPNA3* - Karyopherin Subunit Alpha 3; *NOS1AP* - Nitric Oxide Synthase 1 Adaptor Protein; *NPY* - Neuropeptide Y; *NR3C1* - Nuclear Receptor Subfamily 3 Group C Member 1; *PRKCA* - Protein Kinase C Alpha; *RGS2* - Regulator Of G-Protein Signaling 2; *SLC6A3* - Solute Carrier Family 6 Member 3 (Dopamine transporters); *SLC6A4* - Solute Carrier Family 6 Member 4 (serotonin transporter); *STMN1* - Stathmin 1; *TPH1* - Tryptophan Hydroxylase 1; *TPH2* - Tryptophan Hydroxylase 2; *WWC1* - WW and C2 Domain Containing 1

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Other candidate genes investigated included *APOE, BDNF, NPY* as well as genes involved in the HPA axis (*CNR1, NR3C1, CRHR1, ADCYAP1R1* and *FKBP5*) and the GABAergic system (*GABRA2*). Several of these candidate gene studies have however yielded inconsistent results. This could in part be due to differences in sample population characteristics, methodology used or even be due to small sample sizes (Broekman, Olff & Boer, 2007). Additionally, psychiatric disorders, such as PTSD, are complex, with multiple genes and various biological pathways involved therefore suggesting a complex interaction of several genes involved in this disorder.

1.3.3 Genome-Wide Association Studies in PTSD

Unlike candidate gene studies, genome-wide association studies (GWAS) allow for the investigation of the entire genome in order to detect disease-causing variants. These studies apply a hypothesis-neutral approach by investigating the entire genome for common SNP variation through a case-control study design (Norrholm & Ressler, 2009). However, only a few GWAS (relative to other GWAS in psychiatric disorders) have been performed in PTSD listed in Table 1.3.

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Table 1.3: Genome-wide significant SNPs associated with PTSD as reported by GWAS.

			PTSD cases				Controls			Replication sample	
Reference	**Gene**	**Significant Variant**	**Number**	**Sex**	**Age Mean (SD)**	**PTSD diagnosis**	**Number**	**Sex**	**Age Mean (SD)**	**Number of cases**	**Number of controls**
(Logue, Baldwin, *et al*., 2013)	*RORA*	rs8042149	295 (EA)	~60% male	N/S	CAPS (DSM-IV)	196 (EA)	~60% male	N/S	43 (AA) 100 (AA)	41 (AA) 421 (AA)
(Xie *et al*., 2013)	*COBL*	rs406001	300 (EA)	60.3% female	37.7 (9.8)	CAPS (DSM-IV)	1278 (EA)	35.4% female	38.4 (11.3)	207 (EA)	1692 (EA)
	TLL1	rs6812849	444 (AA)	54.3% female	41.5 (8.7)		2322 (AA)	43.1% female	41.2 (9.3)	89 (AA)	655 (AA)
(Guffanti *et al*., 2013)	*LINC01090*	rs10170218	94 (MA)	All female	52.2 (13.5)	Structured PTSD interview via telephone	319 (MA)	All female	54.3 (15.9)	578 (EA)	1963 (EA)
(Nievergelt *et al*., 2015)	*PRTFDC1*	rs6482463	940 (MA)	All male	23.0 (3.0)	CAPS DSM-IV	2554 (MA)	All male	23.2 (3.5)	313 (EA)	178 (EA)
(Stein *et al*., 2016)	*ANKRD55*	rs159572	497 (AA)	78.8% male	20.4 (3.0)	PCL-C	815 (AA)	82.3% male	21.1 (3.5)	N/A	N/A
	ZNF626	rs11085374	2140 (EA)				2909 (EA)			N/A	N/A

RORA – Retinoid-Related Orphan Receptor A; *COBL* – Cordon-Bleu WH2 Repeat Protein; *TLL1* – Tolloid Like 1; *LINC01090* – Long Intergenic Non-Protein coding RNA 1090; *PRTFDC1* – Phosphoribosyl Transferase Domain Containing 1; *ANKRD55* – Ankyrin Repeat Domain 55; *ZNF626* – Zinc Finger Protein 626 ; EA- European American (excluding Hispanic); ; AA- African American; MA- Mixed American; CAPS- Clinician Administered PTSD Scale (Diagnostic and Statistical Manual of Mental Disorders IV); PCL-C - PTSD Checklist civilian version; N/S – Not Stated; N/A Not Applicable

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The first GWAS in PTSD was performed by Logue *et al*., (2013). The study was somewhat limited in sample size, as GWAS typically requires thousands of samples to achieve genome-wide statistical significance. Nevertheless, they found a specific SNP (rs8042149) located on the retinoid-related orphan receptor alpha (*RORA*) gene to be significantly associated with a lifetime diagnosis of PTSD (Logue, Baldwin, *et al*., 2013). The SNP was however found not significant in two replication studies (listed in Table 1.3) from the same publication using different population groups. Furthermore, an association between the *RORA* (rs17303244) and a fear component of distress (i.e., internalizing factors) was observed using confirmatory factor analysis on a subset of replication samples (N=540) used in the study by Logue *et al*., (2013) (Miller *et al*., 2013). These results could possibly indicate that the *RORA* gene is a risk factor for PTSD. However, due to the conflicting results additional analysis is required.

Another GWAS, by Xie *et al*., (2013), identified a SNP (rs6812849) mapping to the first intron of Tolloid-Like 1 (*TTL1*) gene (Xie *et al*., 2013) to be associated with PTSD in an African American sample group (N=2766) but this did not reach genome-wide significance. Upon additional analysis, two SNPs (rs6812849 and rs7691872) in the first intron of *TLL1* were replicated in an independent sample of European Americans. Furthermore, the Cordon-Bleu WH2 Repeat Protein (*COBL*) gene reached genome-wide significance in a sample of European Americans (Xie *et al*., 2013). Other GWAS investigated risk factors for PTSD identified the lincRNA *LINC01090* (AC068718.1) as a PTSD risk factor (Guffanti *et al*., 2013) in a primarily African American group of woman. This SNP association was only found to be marginally significant in a female European population (578 PTSD cases and 1963 controls).

The two most recent studies to date are also the largest GWAS in PTSD. The first, by Nievergelt *et al*., (2015) identified the phosphoribosyl transferase domain containing 1 (*PRTFDC1*) gene as significant in a mixed sample of Americans (Nievergelt *et al*., 2015). These findings were replicated in an independent sample of trauma-exposed veterans and their intimate partners (313 cases and 178 controls). The second study found rs159572 in the Ankyrin Repeat Domain 55 (*ANKRD55)* gene (which is known to be implicated in inflammatory and autoimmune disorders) to have a genome-wide significance in a sample of African Americans. Additionally, genome-wide significance was found in a sample of 2140 European Americans for a SNP (rs1108537) located in the Zinc Finger Protein 626 (*ZNF626*) gene, believed to be involved in the regulation of RNA transcription. These findings were, however, not replicated in the same publication across the different ethnic groups. In addition to the GWAS mentioned in table 1.3, two GWAS by Wolf *et al*., (2014) and Ashley-Koch *et al*., (2015) failed to detect any SNPs that met genome-wide significance.

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These GWAS are important for identifying neurobiological targets for research in the understanding of disease mechanism and identification of potential drug targets for treatment of PTSD (Almli *et al.*, 2014). However, it is critical that the cases and controls are well-matched for PTSD risk factors in GWAS analyses (Skelton *et al.*, 2012). Unfortunately, GWAS are limited by size of the sample population, relatively small effect sizes, and lack of matching risk factors in case and control study populations.

As GWAS is limited to the identification of common variants, finding rare and more causative variants with greater effects often goes undetected. However, GWAS could provide useful information for NGS-based studies, such as whole genome transcriptomics by identifying genomic regions of interest for further investigation. Most GWAS-identified disease associated variants are localized in non-coding genome regions and likely manifest their influence through the modulation of gene expression. NGS-based methods allow for a more precise quantification of these disease associated variants thereby aiding the detection of their regulatory impact on gene expression (Bahcall, 2015). Therefore, these regulatory variants can be identified by combining global expression profiles from cells or tissues under different conditions with genome-wide genetic variations.

PTSD is a complex disorder and it is therefore likely that numerous variants in several genes, act together to influence the development of this NPD. Therefore, examining the genetic networks involved in PTSD enables for a more practical approach by including several genes and transcription factors involved in gene regulation (Hayden, 2010). Moreover, GWAS enable the detection of heritable gene expression changes but not non-heritable expression changes which include gene expression changes due to epigenetic and/or environmental effects.

1.3.4 Gene Expression Studies in PTSD

Gene expression studies offer an alternative approach to understanding the complex genetic underpinnings of disorders such as PTSD. Unlike GWAS, these studies provide a quantitative method to measure the downstream effects of genetic variations, thereby aiding the identification of possible pathways (and not just rare variants) implicated in PTSD development. Additionally, factors which alter these gene expression patterns could provide insight into the biological underpinnings of PTSD. Several prior studies have identified gene expression level differences between the peripheral blood of PTSD patients and trauma-exposed control individuals thereby identifying potential blood based diagnostic biomarkers for PTSD (Segman *et al.*, 2005; Zieker *et al.*, 2007; Yehuda *et al.*, 2009; Neylan *et al.*, 2011; Glatt *et al.*, 2013; Breen *et al.*, 2015; Tylee *et al.*, 2015).

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In a study by Segman *et al.*, (2005), oligonucleotide microarrays were used to measure gene expression differences in peripheral blood mononuclear cell (PBMC) of trauma survivors directly after a traumatic event (PBMC collected at the emergency room with a mean time between incident and arrival 45±130min) and four months post-trauma. This allowed for the investigation of gene expression differences from immediate onset of a trauma through to the possible subsequent development of PTSD. The results indicated that these psychologically distressed individuals had an overall reduction in expression of transcription activators in PBMC, suggesting that these differences could possibly be explained by a stress-induced reduction of gene expression. A significant increased enrichment ($P<0.0005$) of genes involved in RNA metabolism and processing, as well as nucleotide metabolism was also observed within individual who were subsequently diagnosed with PTSD. The study additionally observed distinct expression signatures for transcripts involved in immune activation, signal transduction and apoptosis. Segman *et al.*, (2005) furthermore identified that the PTSD individuals had significantly dysregulated gene expression of genes involved in the HPA axis (Segman *et al.*, 2005). This was one of the first studies providing evidence that peripheral blood gene expression signatures could in fact be useful in identifying a mental disorder. Thereby enabling the use or more accessible peripheral blood tissue in the investigation of disease process involved in PTSD.

A study by Zieker *et al.*, (2007) similarly identified the dysregulation of stress-response genes in whole blood of PTSD patients with the same environmental trigger (the Ramstein air show catastrophe, 1989) using microarray technology. The study identified downregulation in several immune-related and reactive oxygen species (ROS) genes (*TXR1, SOD1, IL-16, IL-18* and *EDG1*) in PTSD individuals (Zieker *et al.*, 2007). In a microarray study by Yehuda *et al.*, (2009), using a cohort of World Trade Centre attack survivors, dysregulated genes involved in the HPA axis, signal transduction and immune cell functions were identified. With reduced expression of *FKBP5, STAT5B* and major histocompatibility complex class II (MHC-II) molecules observed in PTSD patients compared to trauma-exposed controls (Yehuda *et al.*, 2009).

Additionally, Neylan *et al.*, (2011) found an overall downregulation of gene expression (47 downregulated genes identified ($p < 0.05$)) in the CD14+ monocytes of male PTSD patients. Three of these genes were validated by qPCR including, *PF4, HIST1H2AC* (a histone protein) and *SDPR* (a calcium-independent phospholipid binding protein) (Neylan *et al.*, 2011). These results of an overall decreased gene expression were consistent with that of Segman *et al.*, (2005) with both studies finding an overall reduction in expression of transcription regulators.

Glatt *et al.,* (2013), assessed peripheral blood mononuclear cells in a subset of pre-deployed US marines by comparing marines that subsequently developed PTSD to those who did not develop

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PTSD. The study observed a subset of genes involved in type-1 interferon signaling which was a significantly enriched pathway identified within the dataset. Six of the genes were significantly upregulated (*IFI27, OAS1, OAS2, OAS3, XAF1* and *USP18*) in cases where marines subsequently developed PTSD (Glatt *et al*., 2013). A study from the same group later investigated differential gene expression between post-deployed US marines resulting in the identification of dysregulated genes involved in cellular oxidative stress (Tylee *et al*., 2015).

The microarray studies in PTSD individuals propose that changes in peripheral blood gene expression play a potential role in HPA axis function, glucocorticoid signaling, immune and inflammatory signaling, and the metabolism of reactive oxygen species (ROS). Moreover, dysregulation of genes involved in the management of cellular oxidative stress could represent useful biomarkers for PTSD (Tylee *et al*., 2015).

Gene expression analysis through total RNA sequencing

Whole transcriptome shotgun sequencing better known as RNA-Sequencing (RNA-Seq) is a powerful next-generation sequencing technology (NGS) consisting of both experimental and computational methods (Mortazavi *et al*., 2008; Nagalakshmi *et al*., 2008; Wang, Gerstein & Snyder, 2009). Unlike microarrays, RNA-Seq allows for the generation of unbiased data as the technology is not restricted by probes relying on prior knowledge of the genome. Moreover, this NGS technologies enables the detection of alternative splice sites as well as novel transcripts. The data generated through RNA-Seq can furthermore be stored for further investigation once new genes involved in disease development are discovered.

The transcriptome consists of all RNA transcripts that are transcribed in a cell or in a cell population (Wang, Gerstein & Snyder, 2009) and include both coding and non-coding RNAs. Information gained through the transcriptome differs from that of the exome, as the exome examines all the potential transcripts and not just transcribed RNA. An investigation of the transcriptome is essential for identifying functional elements of the genome that are involved in disease development such as PTSD.

RNA-Seq allows for the investigation of the transcriptome in both a qualitative and quantitative manner. Qualitative RNA-Seq examines expressed transcripts in a given cell population, whilst quantitatively this technology enables the identification of differences in transcription levels between cases and controls. Concerning differential expression analysis, RNA-Seq provides a lower background signal (Wang, Gerstein & Snyder, 2009) detecting both low and high levels of gene

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expression. This contrasts with microarrays which lacks sensitivity for gene expression at very low or very high levels.

Recently, the first RNA-Seq study on PTSD individuals was published by Breen *et al*., (2015) investigated gene expression levels in peripheral blood leukocytes of US Marines pre- and post-deployment to conflict zones. All 188 samples were male and consisted of 47 cases (pre-deployment mean age = 22.15 and post-deployment mean age = 23.14) and 47 controls (pre-deployment mean age = 22.42 and post-deployment mean age = 23.42). Using gene-expression network analyses, the study aimed to integrate expression data across genes into a higher-order context in order to identify groups of genes within a network whose expressions were highly correlated (co-expressed genes). This provided researchers with a robust approach to identify molecular mechanisms in neuropsychiatric disorders such as PTSD. The network analysis resulted in the identification of modules related to haemostasis and wound responsiveness expressed in post-deployment US Marines who did not develop PTSD. The study moreover observed dysregulated innate immune module (interferon (IFN) signalling) to be associated with the development of PTSD with the top five hub genes identified for post-deployment as *IFI35, IFIH1, PARP14, RSAD2* and *UBE2L6*) and pre-deployment as (*DTX3L, IFIH1, IFIT3, PARP14* and *STAT2*) (Breen *et al*., 2015).

Gene expression studies are integral to reveal the biological underpinnings of disease through the identification of thousands of genes associated to diseases. This approach could lead to the discovery of disease blood biomarkers, possibly improving the clinical diagnosis of psychiatric disorders such as PTSD. The current gene expression study is to our knowledge the first RNA-Seq study investigating PTSD based on civilian trauma. The study will also assist in the generation of a complete and unique catalogue of coding sequence variation and associated frequency information from South African transcriptomes.

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The current study

Significance of study

The current research project was conducted as part of a larger interdisciplinary South African Medical Research Council (MRC) Flagship-funded project, known as SHARED ROOTS. The MRC flagship project intends to examine genomic, neural, cellular and environmental signatures that are common between neuropsychiatric diseases (NPDs) such as PTSD and cardiovascular disorders (CVD), as defined by metabolic syndrome (MetS). Numerous studies have shown the occurrence of MetS in individuals with PTSD (Wentworth *et al.*, 2013). By combining genomic, transcriptomic, epigenetic and neuroimaging data, the flagship project aims to identify the mechanistic pathways involved in this comorbidity.

One of the aims of the SHARED ROOTS project is the application of blood-based whole transcriptome in a subset of PTSD patients and trauma-exposed controls to identify a set of differentially expressed genes (DEG) between patients with and without MetS.

An important step in achieving this, is a preliminary investigation in identifying a set of DEGs through whole genome transcriptomics between PTSD patients and trauma-exposed controls, where MetS phenotype is excluded. This approach allows for a concerted view of the underlying molecular mechanisms involved in PTSD specifically, without phenotypic complexity associated with MetS.

The present study will focus on the genetic mechanisms involved in PTSD using a hypothesis-generating approach, facilitating the discovery of novel gene candidates and possible molecular pathways implicated in the disease. Such an unbiased approach can be gained using whole-genome transcriptomics approach, such as RNA-Seq, which allows for the identification of potential biological pathways involved in neuropsychiatric diseases by use of a quantifying gene expression approach.

The research presented in this thesis therefore aims to address the above by identifying differential expression between PTSD patients and trauma-exposed controls using RNA-Seq to explore links between the identified subset of genes and their known functions, phenotypes, their involvement in known disease pathway and gene-gene interaction associated with clinical phenotypes (such as: hyperarousal, insomnia, agitation, avoidance, derealisation, dissociation, and depression) of PTSD, using ontology variant prioritization strategy. Potentially contributing to the identification of the underlying molecular mechanism of PTSD.

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Thesis aims and objectives

The research aimed to investigate the molecular mechanisms involved in PTSD on a whole genome transcriptomic level by carrying out differentially expressed gene set analysis on PTSD patients and trauma-exposed controls.

Objectives:

I. To identify a set of genes that are differentially expressed in PTSD patients compared to trauma-exposed controls.

II. To investigate molecular functions, phenotypes, pathways and gene-gene interactions dysregulated by the differentially expressed gene set in PTSD patients using an ontology driven gene prioritization strategy using PTSD-specific BioOntological Relationship Graph Database.

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CHAPTER 2 : METHODOLOGY

2.1 Ethical considerations

The present preliminary study forms part of the MRC flagship study SHARED ROOTS for which ethics approval was granted by the Health Research Ethics Committee of Stellenbosch University. Human research was conducted according to the ethical guidelines and principles of the Declaration of Helsinki, SA Good Clinical Practice Guidelines and the MRC Ethical Guidelines for Research (Ref: N13/08/115). Informed written consent was obtained from all participants. Raw data was captured into a secure, anonymised central RedCap database. The database could only be accessed by study personnel and students involved in the SHARED ROOTS study. The identities of participants was anonymised and not directly linked to information captured within the dataset.

2.2 Subject recruitment

The present study recruited South African mixed ancestry individuals, aged 18 years and above, from the Western Cape Province. Mixed ancestry individuals in South Africa refers to a multiracial ethnic group (Coloured individuals) with genetic contributions from various population groups including Europeans, South Asians, Indonesians and the sub-Saharan Bantu population (Patterson *et al.*, 2010). The unique composition of this admixed population provides an opportunity to discover novel susceptibility alleles underlying many multifactorial diseases, facilitating the discovery of new gene associations with disease traits (de Wit *et al.*, 2010).

Purposive sampling was employed in the recruitment of all study participants.

2.2.1 Clinical assessments and questionnaires

All participants were consulted for at least two visits at the Faculty of Medicine and Health Sciences, Tygerberg. The Clinician Administered Posttraumatic Stress Disorder Scale for DSM-5 (CAPS-5) was used to determine PTSD diagnostic status (Weathers, Blake, *et al.*, 2013a) by a clinician with expertise in the field of psychiatry. Moreover, the Life Events Checklist for the DSM-5 (LEC-5) (Weathers, Blake, *et al.*, 2013b) was utilised to assess for a lifetime history of exposure to potentially traumatic events, whilst the Childhood Trauma Questionnaire (CTQ) (Bernstein & Flink, 1998)

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screened for a history of child abuse and neglect. Self-report PTSD symptom severity was determined with the PTSD Checklist for DSM-5 (PCL-5) (Weathers, Litz, *et al*., 2013) in all trauma-exposed participants. Current and lifetime psychiatric disorders were accounted for by use of the MINI International Neuropsychiatric Interview, version 6.0 (MINI) (Sheehan *et al*., 2009). Demographic data were obtained with a demographic questionnaire, personal and family history of medical and psychiatric illness and previous and current medication use with a medical history questionnaire. All data was captured in a RedCap database (Harris *et al*., 2009).

2.2.2 Selection criteria

A total of 50 age-matched trauma-exposed participants, identified by the LEC-5, were included in the present sub-study to assess for a lifetime history of exposure to potentially traumatic events. Twenty of these participants were diagnosed with PTSD based on CAPS (CAPS ≥ 28), whilst the additional 30 trauma-exposed participants were used as controls. Of the thirty trauma-exposed controls, only 20 controls had completed the CAPS (CAPS ≤ 5) assessment. The remaining ten trauma-exposed controls were included as controls for another cohort in the SHARED ROOTS study (Parkinson's disease), where CAPS was not administered (see Chapter 3, Section 3.1.1). This was however the only difference in the methodology as all procedures between the cohorts' of trauma-exposed controls were the same. Data from the PCL-5 assessment was captured for all 50 of the participants.

As part of the exclusion criteria, participants with MetS or illicit drug use in the past six months were omitted (substance use omitted based on a medical history questionnaire). Relatives of participants with serious mental disorders such as schizophrenia and bipolar disorders were excluded. Psychiatric comorbid disorders such as major depressive disorder (MDD) were not excluded in five participants due to the high comorbidity between MDD and PTSD. This made it difficult to completely exclude MDD from the PTSD patient.

The present study only included mixed ancestry, female participants as women have a twofold higher risk for developing PTSD.

2.2.3 Sample collection

A 2.5 ml PAXgene® Blood RNA Tube (QIAGEN®, Hilden, Germany) was used for collection of whole blood from participants. Samples were logged with a lab identification number at the research laboratory in the Division of Molecular Biology and Human Genetics at the Faculty of Medicine and

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Health Sciences, Stellenbosch University and kept at room temperature between two to 24 hours. The PAXgene Blood RNA tubes were then transferred to a -20 °C freezer for 24 hours and thereafter stored at -80 °C until RNA isolation as per manufacturer's instructions.

2.3 Whole blood RNA extraction

The PAXgene® Blood RNA kit (QIAGEN®, Hilden, Germany) was used as per manufacturer's instructions to isolate totalRNA (tRNA) from PAXgene® Blood RNA Tubes (QIAGEN®, Hilden, Germany) containing 2.5 ml of peripheral blood. All samples were extracted at an RNase-free bench in a research laboratory at the Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University. All surfaces were cleaned with 70% ethanol in combination with RNaseZap® (Ambion, Inc., Austin, Texas) for a clean, sterile RNA extraction environment. Samples were thawed for a total of 24 hours before total RNA extraction.

Modifications were made to the final elution step of the protocol by re-pipetting the 40 µl of RNA elute (RNA eluted with Buffer BR5) onto the PAXgene RNA spin column membrane to increase RNA concentration. Therefore, leaving an end volume of 40 µl instead of 80 µl as specified by the protocol. A total of 4 µl of each sample was pipetted into PCR tubes for subsequent quality and quantity assessment on the NanoDrop™ and Agilent Bioanalyzer. The remainder of the isolated RNA was stored in a -80 °C freezer for RNA-Seq analyses. A maximum of four samples were isolated at a time as to limit cross-contamination of samples.

2.4 Quality and quantity assessment of extracted RNA

The quality and quantity of extracted RNA was analysed using two different methods. First, RNA yield was quantified by absorbance readings at 260 nm and 280 nm using the NanoDrop™ 2000c Spectrophotometer (Thermo Scientific, Delaware, USA) allowing for an estimation of the quality of RNA. However, free nucleotides and other organic compounds used in the extraction of RNA will also absorb UV light near 260 nm, resulting in an overestimation of the RNA concentration. For this reason, a second quantity evaluation was necessary.

Quantity and quality assessments were performed on the 2100 Bioanalyzer platform (Agilent Technologies, California, USA) at the Central Analytical Facilities (CAF) of Stellenbosch University,

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according to manufacturer's instructions. RNA integrity numbers (RIN) were the assessed. Samples with RIN values higher than 7 were sent for RNA-sequencing (as per NXT-Dx (Ghent, Belgium) instructions). This value indicates the range of RNA degradation from 1-10, with a value of 1 indicative of total degradation and a value of 10 representing intact RNA. A RIN value of 7 and above is considered as high quality, intact RNA (Schroeder *et al.*, 2006).

2.5 RNA-Sequencing

The 50 RNA samples, with RIN values above seven, were sent on dry ice to the service provider NXT-Dx (Ghent, Belgium) for Whole RNA sequencing. All samples were diluted to contain a minimum of 500 ng of RNA per sample with the volume varying between 15-50 µl as per sequencing company's instruction. TruSeq® stranded total RNA kit (Illumina®, California, USA) was used as per manufacturer's instructions. Samples were multiplexed and paired-end (PE) library clusters were then generated using the cBot (Illumina, California, USA) as platform. The DNA clusters, contained in the flow cell, were then sequenced using the Illumina HTSeq 4000 as platform at a sequencing depth of 50 million PE reads per sample with a read length of 50 base pairs.

2.5.1 Overview of RNA-Seq workflow

Total RNA was purified by the removal of ribosomal RNA (rRNA) using a combination of biotinylated, target specific oligos and Ribo-Zero™ beads. After purification, remaining RNA was fragmented (Figure 2.1 A) followed by random priming to allow for first strand cDNA synthesis by reverse transcriptase. Thereafter E. coli DNA polymerase I and RNase H was utilised for cDNA second strand synthesis, incorporating dUTPs within the second strand to generate double stranded (ds) cDNA (Figure 2.1 B). A single adenine (A) base was then added to the blunt-end ds cDNA fragment (Figure 2.1 C) allowing for the ligation with a thymine (T) overhang located at the 3'end of the adapter (Figure 2.1 D). The ds cDNA product was then denatured, purified and amplified by PCR using primers complementary to adapters to create the final cDNA library (Figure 2.1 E). In this PCR process, only DNA fragments that correctly annealed to primers and incorporated dTTPs during strand synthesis were amplified. The dUTP's incorporated at second strand synthesis therefore allowed for the preparation of strand-specific libraries as DNA polymerase is unable to extend dUTP bases during the PCR reaction. These libraries thus only contain strands of the original RNA template allowing for the generation of enriched cDNA libraries.

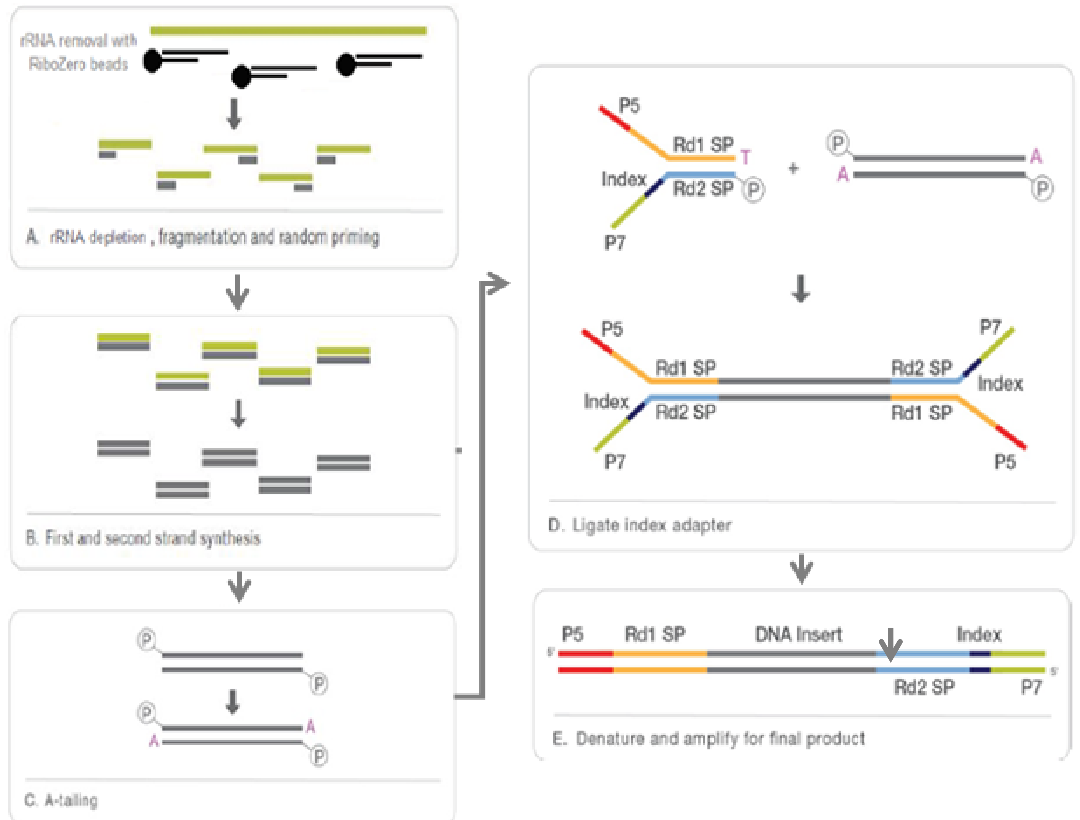

Figure 2.1: The TruSeq® stranded total RNA library preparation workflow. A) During library preparation total RNA was purified, removing ribosomal RNA (rRNA). **B)** The remaining RNA was fragmented followed by random priming for first strand cDNA synthesis. Second strand synthesis then incorporates dUTPs generating double stranded (ds) cDNA. **C)** A single adenine (A) base was added to the blunt-end ds cDNA fragment allowing **D)** the ligation of a thymine (T) overhang. **E)** The ds cDNA product was then denatured, purified and amplified by PCR creating the final cDNA library used in cluster generation (Adapted from http://www.illumina.com).

Samples were multiplexed, which allowed for the pooling of multiple samples in a single sequencing reaction through use of unique index labels located on primers. Thereafter, PE library clusters were generated using the cBot (Illumina, California, USA) as platform. The cluster generation occurs on a flow cell containing lanes of nanowells filled with oligos complementary to library adapters. During cluster generation, a novel exclusion amplification method insures that only a single DNA template is able to bind to form a cluster within a single nanowell. Moreover, polyclonal cluster formation is prevented due to the rapid amplification of libraries after binding to an oligo primer. Insuring a monoclonal cluster is formed within each nanowell leading to a high cluster of cells originating from a single template.

The bound libraries were then extended by polymerases and the original template is washed away leaving only the newly synthesized strand covalently attached to the flow cell surface. This was followed by bridge amplification where the single stranded molecule hybridizes to an adjacent,

complementary primer extending to a double-stranded bridge through polymerase. The double-stranded bridge was then denatured resulting in two copies of covalently bound single-stranded templates. The process was then repeated in multiple cycles until several bridges were formed. Reverse strands were then cleaved and washed away leaving a cluster consisting only of forward strands, blocking the free 3'ends to prevent unwanted DNA priming. A read 1 sequencing primer was then hybridized to the adapter sequence incorporated in the template strand.

The DNA clusters, contained in the flow cell, were then sequenced using the Illumina HTSeq 4000 as platform. This platform employs a process known as sequencing by synthesis (SBS), were single bases are detected as they are incorporated to the template strand (Figure 2.2)

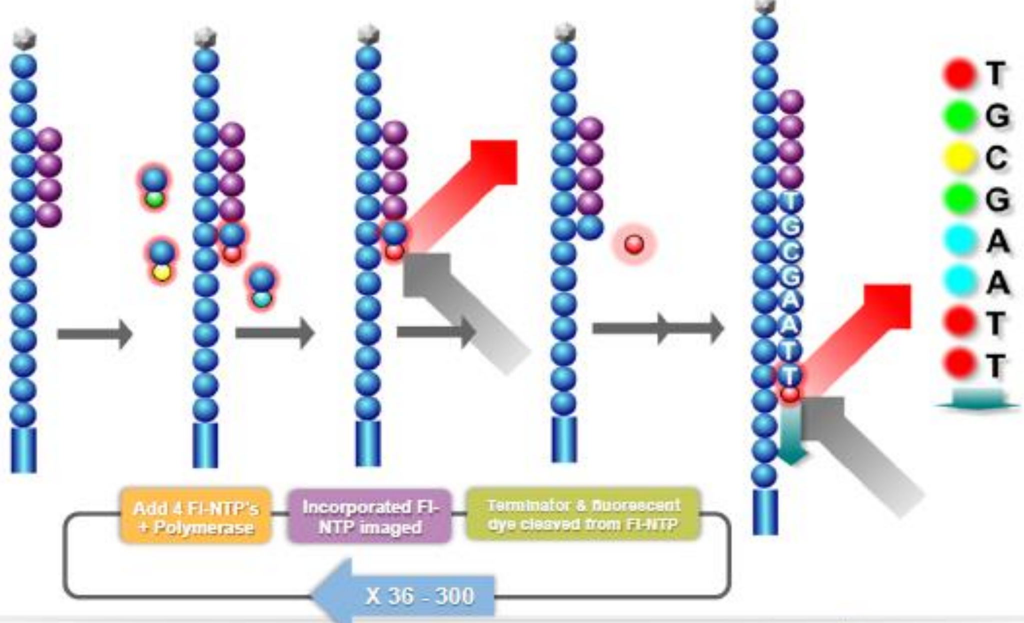

Figure 2.2: Illustration of sequencing by synthesis (SBS) used by the Illumina HTSeq platform. A complementary fluorescently labelled nucleotide is incorporated to a template strand, when bound a fluorescent dye is cleaved off and excited by a light source. This emits a signal for each of the four possible nucleotides determining the base call of the template strand accordingly. In specific clusters all identical strands are read simultaneously for a parallel process (read length determined by number of cycles). The read product is then washed away and an index read is incorporated identifying the template strand (Adapted from http://www.illumina.com).

In this sequencing method, a complementary fluorescently labelled nucleotide is incorporated to the template strand. When a nucleotide binds to the template strand a fluorescent dye is cleaved off and excited by a light source. This emits a characteristic signal for each of the four possible nucleotides determining the base call accordingly. In a specific cluster, all the identical strands are read simultaneously allowing for a parallel process where the number of cycles determines the length of

the read. Afterwards the read product was washed away followed by the incorporation of a specific index read (index 1) to the first reads generated (forward template).

In paired end sequencing the 3'ends of the template strands are unblocked, hybridizing to the second oligo on the flow cell. A second index read (index 2) was then incorporated and the read product washed away. Polymerases extended the second oligo forming a double stranded bridge. Bridges were then linearized and the original forward template is cleaved off and the free 3' ends of the reverse template blocked to prevent unwanted DNA priming. A read 2 sequencing primer was then hybridized to the adapter sequence followed by SBS of the second read (reverse template). Sequences from the pooled sample libraries were then separated based on their unique indexes (Figure 2.3).

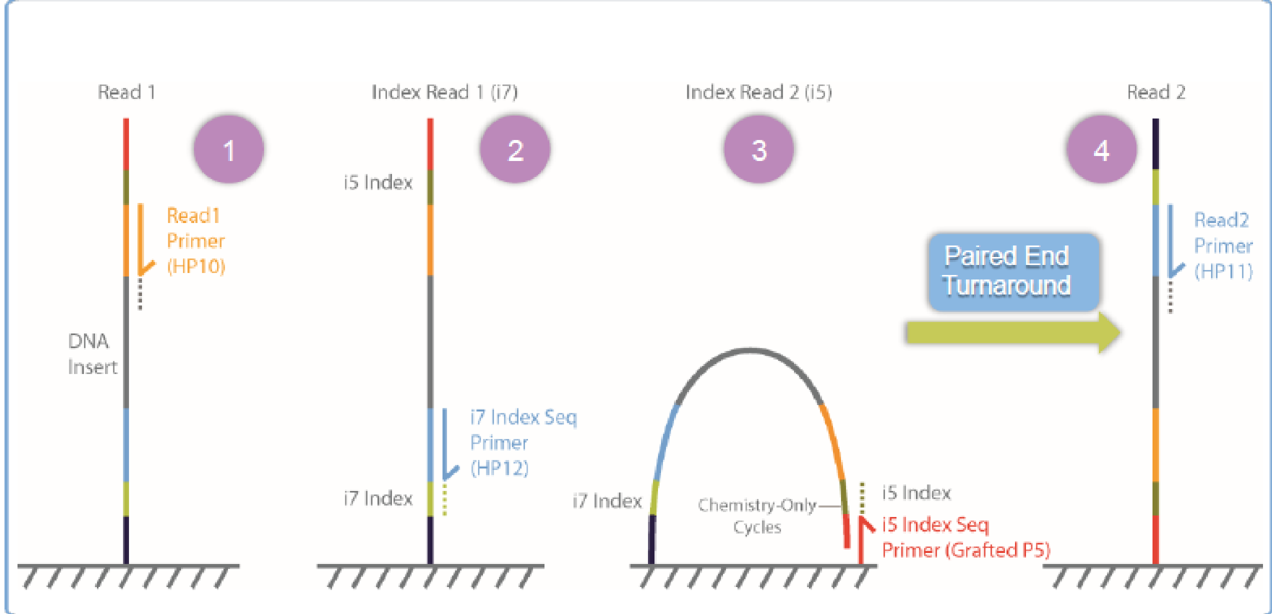

Figure 2.3: Illustration of how paired-end (PE) reads are generated through sequencing by synthesis. 1) Sequencing reads are generated for a forward template using SBS. **2)** a sequencing index primer is added incorporating the sequence into read 1. The 3'ends of the template strands are unblocked, hybridizing to the second oligo on the flow cell. A second index read (index 2) is incorporated and the read product washed away. **3)** Polymerases extended the second oligo forming a double stranded bridge. **4)** Bridges linearize, the original forward template is cleaved off and the free 3' ends of reverse templates are blocked (prevent unwanted DNA priming). The read 2 sequencing primer is hybridized to the adapter sequence followed by SBS of the second read (reverse template). Sample libraries are pooled and separated based on their unique indexes (Adapted from http://www.illumina.com).

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2.6 Data processing

RNA-Seq studies generate large amount of data, requiring extensive computational capacity for storage, processing and downstream analysis. To leverage the analytical and computational needs, the South African National Bioinformatics Institute (SANBI), as the national bioinformatics core in the country and collaborator on the SHARED ROOTS project, provided computational infrastructure and expertise, where data processing and downstream analysis were carried out using state of the art tools and technology recommended by international experts in the field.

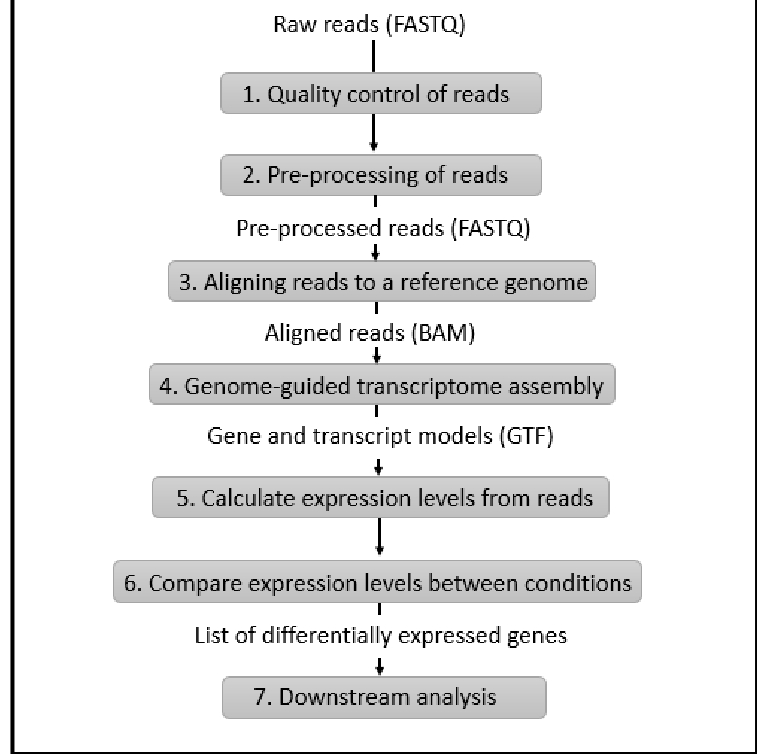

Figure 2.4: Schematic representation of bioinformatics analyses workflow used to identify differentially expressed genes between PTSD patients and trauma-exposed controls. Pipeline used to identify differentially expressed genes from raw data generated by RNA-Seq.

2.6.1 Quality control and trimming using FastQC and Trim-Galore

The FastQC tool, v 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used for quality assessment of the generated reads from the RNA-Seq data. The FastQC tool provides output information such as on the average read length, GC content as well as the presence and abundance of contaminating sequences. All raw sequence reads were of high quality with read length of 50 nucleotides.

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As majority of the reads passed the quality control using FastQC, only index trimming was carried out. Trim-Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), a wrapper tool for Cutadapt (https://cutadapt.readthedocs.io/) and FastQC, was used to trim the 8bp indexes at the 3'end of both the forward (R1) and reverse (R2) paired end reads of FastQ files before being mapped to the reference genome. These unique index labels allowed for the tagging of several reads, pooling multiple samples in a single sequencing reaction (Appendix 1 Table I.1).

2.6.2 Alignment to reference genome

Hierarchical indexing for spliced alignment of transcripts (HISAT2) (https://ccb.jhu.edu/software/hisat2/) tool was used for alignment of RNA-Seq reads to the human reference genome (GRCh38). The goal of the alignment process is to locate the origin of each sequenced read within the reference genome. Additionally, SAMtools (www.htslib.org/) were used to convert the mapped reads from SAM format to BAM format as well as sorting of the mapped reads based on gene names (as a prerequisite for read-count by HTSeq).

2.6.3 Calculating expression levels of reads using HTSeq

The HTSeq-count tool (http://www-huber.embl.de/HTSeq) was utilized to count the number of reads mapped to each gene coordinates. Input files required for the tool included the aligned reads, ordered according to gene names in BAM format, and a gene transfer format (GTF) tab-delimited file containing the coordinates of exon boundaries. The output text file, containing the gene name counts, was then used as input for differential gene expression analysis.

2.7 Differential expression analysis using DESEQ2

Differential expression analysis entails the identification of differentially expressed transcripts of genes, which differ significantly between cases and controls. This allows for the identification of upregulated and downregulated genes using statistical testing. To calculate the levels of differentially expressed transcripts in our study cohort, DESEQ2 tool (https://bioconductor.org/packages/DESeq2) was used in R environment. DESEQ2 employs count data for a more quantitative analysis of differential expressed transcripts. DESEQ2 uses Benjamin Hochberg adjusted p-value (FDR), which is the smallest significance level at which a particular comparison will be declared statistically

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significant as part of the multiple comparison testing. An adjusted p-value cutoff of less than 0.05 was used to identify significantly differentially expressed genes (DEGs), whilst a log fold change of ≥ 0.25 and ≤ -0.25 was used for the identification of up- or down- regulated genes, respectively.

2.8 Using BioOntological Relationship Graph Database to identify gene-disease links

While it is now fairly routine to identify genes bearing deleterious mutations or which are differentially expressed, it is not always obvious to identify the strongest set of candidates for involvement in a disease or phenotype of interest. Accordingly, assessing candidate genes in context of existing biomedical knowledge and their known biomolecular functions is an important step in producing a manageable set of genes for further validation and exploration.

To determine biologically relevant genes from the list of significantly DEGs in our study, an ontology-driven variant prioritisation approach, using an anxiety disorder-specific BioOntological Relationship Graph (BORG) database was utilized. The semantic database allows for the biological contextualisation of a set of prioritized genes by use of a large on-disk virtual mind map, which integrates millions of biological and biomedical facts about human, mouse and rat genes into a single semantic network. Several bio-ontologies and ontology term annotated genes are used to model existing biomedical knowledge in the database:

I. Gene Ontology (GO) terms based on annotations published by the GO consortium (Berardini *et al*, 2010).
II. Disease Ontology (DO), which indicates known involvement of genes in the disease of interest (http://www.geneontology.org/).
III. Human Phenotype Ontology (HPO) (www.human-phenotype-ontology.org), terms based on the phenotypes that are documented to be associated with human genes in the OMIM database (https://www.omim.org/).
IV. Mammalian Phenotype Ontology (MPO), uses information gathered from the Jackson lab (http://www.informatics.jax.org/) based on gene knockout models using mouse and rat. This ontology uses standardised terms for phenotype observed in these mice and rat models when a gene is knocked out allowing for a transitive association with human genes (http://www.informatics.jax.org/glossary/mammalian_phenotype_ontology).
V. Pathway Ontology (PW), which consists of biological and disease pathways involved in a particular gene (http://bioportal.bioontology.org/ontologies/PW).

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To build a semantic model, anxiety disorder was specified as the disease ontology term and a set of terms, which characterises the phenotype of interest was created based on a list of specific hierarchical, ontology terms, which link concepts (nodes) and their relationships (edges) to the disease. Anxiety disorder disease ontology (DO) term was selected on a hierarchical basis, with more specialised terms, such as "PTSD", being captured as part of the broader anxiety disorder terms. This facilitated the discovery of biologically plausible genes which might have been otherwise missed when using more specialised term such as "PTSD"). This cross-ontological mapping allows for the identification of genes based on gene functions, phenotypes and pathways that are involved, or predicted to be involved, in anxiety disorders and by extension in PTSD. This approach may uncover non-obvious, yet biologically plausible and literature-supported genes associated to disease. The final list of GO, HPO and MPO terms used to build the anxiety disorder model in BORG (referred to as "anxiety BORG" throughout the present study) is shown in Table 2.1.

Effectively, the system evaluates the following questions specific to our study:

1) Is the differentially expressed gene known or predicted to be involved in the disease of interest?
2) Does the gene have a function that coincides with the pathology?
3) Is the gene in a pathway associated with the disease?
4) Does the human gene or an animal knockout of the gene cause the disease or a hallmark phenotype of the disease?
5) Is the gene expressed in the tissue or organ of interest?
6) Does the gene product physically interact with a protein that is encoded by a known disease gene?

Using "guilt-by-indirect-association" the database finds links between known disease gene previously identified by other experimental studies and large GWAS data and anxiety disorder, which allows for identification of a potential subset of novel genes linked to disease based on indirect associations, which might have been otherwise missed (Figure 2.5).

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Table 2.1: Ontology terms selected to transitively link genes to anxiety disorder and by extension to PTSD in the BORG semantic database.

Ontologies	BORG Disease Terms	
Gene Ontology	GO:0001662	behavioral fear response
	GO:0002122	fear-induced aggressive behavior
	GO:0004972	NMDA glutamate receptor activity
	GO:0014056	regulation of acetylcholine secretion, neurotransmission
	GO:0014062	regulation of serotonin secretion
	GO:0017146	NMDA selective glutamate receptor complex
	GO:0019722	calcium-mediated signaling
	GO:0031961	cortisol receptor binding
	GO:0032899	regulation of neurotrophin production
	GO:0032902	nerve growth factor production
	GO:0035259	glucocorticoid receptor binding
	GO:0035640	exploration behavior
	GO:0046928	regulation of neurotransmitter secretion
	GO:0050780	dopamine receptor binding
	GO:0051378	serotonin binding
	GO:0051610	serotonin uptake
	GO:0051611	regulation of serotonin uptake
	GO:0051614	inhibition of serotonin uptake
	GO:0051866	general adaptation syndrome
	GO:0097114	NMDA glutamate receptor clustering
	GO:2000822	regulation of behavioral fear response
Human Phenotype	HP:0000722	Obsessive-compulsive behavior
	HP:0000723	Restrictive behavior
	HP:0000733	Stereotypic behavior
	HP:0000739	Anxiety
	HP:0008770	Obsessive-compulsive trait
	HP:0100851	Abnormal emotion/affect behavior
	HP:0100852	Abnormal fear/anxiety-related behavior
Mammalian Phenotype	MP:0001362	abnormal anxiety-related response
	MP:0001363	increased anxiety-related response
	MP:0001364	decreased anxiety-related response
	MP:0001454	abnormal cued conditioning behavior
	MP:0001469	abnormal contextual conditioning behavior
	MP:0002063	abnormal learning/memory/conditioning
	MP:0002065	abnormal fear/anxiety-related behavior
	MP:0002797	increased thigmotaxis
	MP:0002803	abnormal operant conditioning behavior
	MP:0002806	abnormal conditioned emotional response
	MP:0003106	abnormal fear-related response
	MP:0003360	abnormal depression-related behavior
	MP:0006299	abnormal latent inhibition of conditioning behavior
Pathway	PW:0000240	neuropsychiatric disease pathway
	PW:0000272	neuron-to-neuron signaling pathways
	PW:0000274	neuron-to-neuron signaling pathway via the chemical
	PW:0000389	altered signaling pathway pertinent to the brain and nervous
	PW:0000442	norepinephrine metabolic pathway

	PW:0000448	neuropeptide Y metabolic pathway
	PW:0000450	neurotensin metabolic pathway
	PW:0000493	corticotropin-releasing hormone signaling pathway
	PW:0000569	cortisol signaling pathway
	PW:0000571	neurotrophic factor signaling pathway
	PW:0000572	brain-derived neurotrophic factor signaling pathway
	PW:0000755	benzodiazepine drug pathway
	PW:0000782	glucocorticoid signaling pathway
	PW:0001140	calcium/calcium-mediated signaling pathway
	PW:0001141	calcium signaling pathway via the calcium-sensing receptor

NMDA - N-methyl-D-aspartate

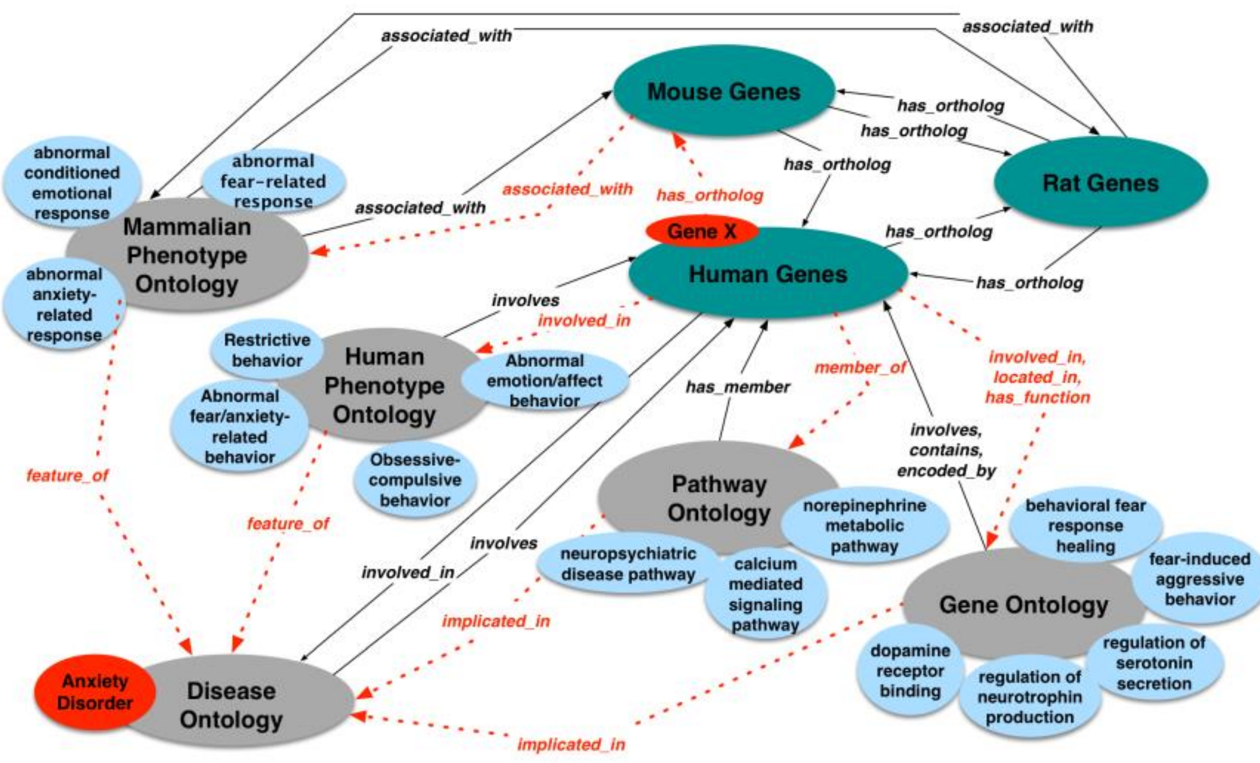

Figure 2.5: BioOntological Relationship Graph (BORG) database schema. The diagram represents semantic relationships of known gene and disease from biomedical databases and published curated relationships (linked by black arrows). Red dotted arrows indicate cross-ontological mapping of 'novel' genes to disease via pathways (PO), phenotypes (HPO and MPO) and functions (GO) that are associated to anxiety disorder.

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2.9 Gene set enrichment analysis through Enrichr

Gene set enrichment analysis was performed on all differentially expressed up and downregulated genes identified by DESEQ2 using the Enrichr tool (http://amp.pharm.mssm.edu/Enrichr). Enrichr functions as an integrative web-based tool that provides summaries of an input gene lists collective functions, against prior knowledge gene-set libraries. This type of analysis allows for the grouping of DEGs based on their functional similarities allowing for the biological interpretation of the 556 DEGs identified by DESEQ2. The tool consists of gene-set library from a variety of tools including, but not limited to, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.genome.jp/kegg/), WikiPathways (http://www.wikipathways.org), Online Mendelian Inheritance in Man (OMIM) (https://www.omim.org/) disease and ontology library gene-sets such as biological process, molecular function and cellular component as part of GO.

2.10 Identifying gene set co-expression using COXPRESdb

COXPRESdb (http://coxpresdb.jp) was used to explore potential co-expression between the prioritized set of DEGs obtained from our BORG analysis. This database for animal species employs both DNA-microarray and RNA-Seq based expression data with multiple quality assessment systems in place to identify co-expressed genes. Through this type of approach, we prioritised genes based on their known functions associated with the PTSD. By use of the EdgeAnnotation tool all possible co-expression pairs for the biologically relevant query genes were determined, reporting both on mutual rank (MR) and correlation values between the genes. The MR values which represents a geometric averaged correlation rank calculated between the two directional ranks were used (i.e. the rank of gene B from gene A versus the rank of gene A from gene B calculated by $MR_{(AB)} = \sqrt{(Rank_{(A\rightarrow B)} \times Rank_{(B\rightarrow A)})}$). Thereafter, co-expression gene networks were drawn with the NetworkDrawer tool to identify highly correlated gene pairs based on MR values.

2.11 Tissue expression identification using the GTEx portal

Tissue expression of co-expressed genes of interest were examined through the Genotype-Tissue Expression (GTEx) (http://gtexportal.org) portal, providing insight on healthy human gene expression patterns in multiple tissues. The database was used to determine the level of gene expression present within tissues of interest associated to PTSD such as brain in comparison to whole blood. Brain tissues reported included, the amygdala, anterior cingulate cortex, caudate, cerebellar hemispheres,

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cerebellum, cortex, frontal cortex, hippocampus, hypothalamus, nucleus accumbens, putamen, spinal cord and substantia nigra.

2.12 Summary of methodology workflow

A diagram providing a summary of the methodology workflow used in the current study is indicated in Figure 2.6.

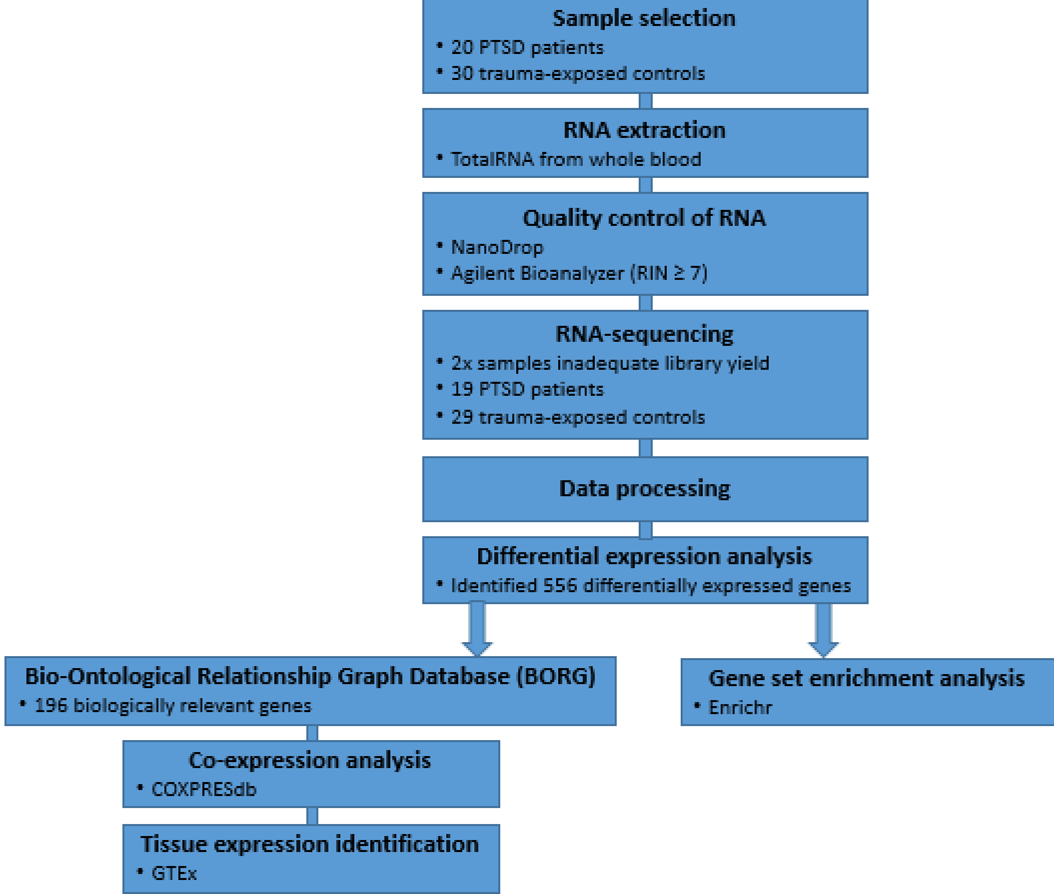

Figure 2.6: Flow diagram providing a summary of the methodology used in an RNA-Seq study investigating differential expression between PTSD patients and trauma-exposed controls. Total RNA (tRNA) from 50 female, mixed ancestry participants (n = 20 cases and n = 30 controls) was isolated from whole blood and subsequently quality controlled through two methods (Using the NanoDrop and Agilent Bioanalyzer). High quality (RNA integrity number ≥ 7) tRNA samples were sent for RNA-Seq. Two samples yielded insufficient sequencing libraries leading to the bioinformatics processing and differential expression analysis of 48 samples (19 cases and 29 controls). Expression analysis identified 556 which were analysed through *Enrichr* identifying the general functions and pathways enriched for the gene set. Additionally, the 556 DEGs were analysed through BORG, identifying 196 gene biologically relevant based on ontology terms linked to anxiety and by extension to PTSD. This was followed by co-expression analysis identifying genes with similar expression patterns and tissue expression identification.

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CHAPTER 3 : RESULTS

3.1 Subject Recruitment

3.1.1 Clinical and demographic data

Fifty participants (20 cases and 30 controls) were recruited for the present study of which only 48 samples (19 cases and 29 controls) were sequenced. This was due to the low RNA concentration of two samples (low concentrations possibly occurred when dilution were made) which led to low and insufficient cDNA library yield for RNA-Seq. Additionally, ten of the 29 trauma-exposed controls selected were initially included as controls for another cohort in the SHARED ROOTS study (Parkinson's disease). These additional ten trauma-exposed controls were included in the current study in order to observe a stronger differential expression pattern between the trauma-exposed controls compared to the PTSD group. All participants underwent the same procedures with the exception of the CAPS which was not administered to the ten initial trauma-exposed controls from the Parkinson's disease cohort. Table 3.1 summarizes the clinical and demographic data captured for the 48 sequenced samples.

Table 3.1: Clinical and demographic data of 48 samples (PTSD cases vs trauma-exposed controls) sequenced through RNA-Seq.

	PTSD (n=19)	Control TE (n=29)	p-value
Age in years, mean (SD)	40.00 (12.01)	42.00 (14.68)	0.674
CAPS-5 total score, median (IQR)	32.00 (31.00-44.00)	0.00 (0.00-1.00) (n = 19)	-
Time since index trauma (months), median (IQR)	96.00 (12.00-240.00)	36.00 (12.00-114.00)	0.399
Number of different types of traumatic experiences on LEC-5, mean (SD)	5.95 (2.64)	2.93 (1.85)	0.000154*
CTQ total score, median (IQR)	43.00 (35.00 - 74.00)	33.00 (30.50- 46.50)	0.025*
PCL-5 total score, median (IQR)	50.00 (44.00-58.00)	5.00 (0.00-12.00)	1.02E-13*

CAPS-5 - Clinician Administered Posttraumatic Stress Disorder Scale for DSM-5; CTQ - Childhood Trauma Questionnaire; IQR- inter quartile range; LEC-5 - Life Events Checklist for DSM-5; PCL-5 - PTSD Checklist for DSM-5; SD – standard deviation; TE – trauma-exposed
*significant ($p < 0.05$)
(p-value based on independent t-test ($p < 0.05$) where equal variances were not assumed using SPSS)

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All 48 samples were mixed ancestry females with no significant difference between the age of PTSD patients and trauma-exposed controls (p = 0.674). Moreover, there was no significant group difference in the time since the index trauma (p = 0.399). The CAPS total severity score data demonstrated a median score of 32.00 (IQR: 31.00-44.00) and 0.00 (IQR: 0.00-1.00) for PTSD patients and controls respectively, (CAPS scores were only available for 65.50% of the trauma-exposed controls). However, the PCL total scores were available for all 48 participants indicating that PTSD patients had a median score of 50.00 (IQR: 44.00-58.00) compared to 5.00 (IQR: 0.00-12.00) for the controls. The number of traumatic experiences, based on the LEC-5 scores, showed a significant difference between groups (p = 0.000154), with cases experiencing almost double the number of traumatic events experienced by controls. The median CTQ total score was significantly (p = 0.025) higher in patients (43.00 [IQR: 35.00-74.00]) than in controls (33.00 [IQR: 30.50-46.50]).

3.2 Quality and quantity assessment of extracted RNA

The NanoDrop™ spectrophotometer was used in identifying RNA yield after extraction. Thereafter, samples were analysed using the Agilent Bioanalyzer, which offers a higher reliability in quantitative measurements than that of the spectrophotometer. Qualitatively, the Bioanalyzer reported that all 50 extracted samples had RIN values higher than seven. These samples were subsequently sent for RNA-Seq. Table 3.2 provides a summary of the RNA quantity and quality of the 50 samples sent for RNA-Seq. Figure 3.1 illustrates a representative Bioanalyzer report of a sample with both a high RIN value and an adequate RNA concentration for next-generation RNA-Seq.

Table 3.2: Summary of Bioanalyzer results for sample sent for RNA-Seq. A minimum of 500 ng of RNA in 15-50 µl was sent for sequencing. Additionally, a RIN ≥ 7 on the Agilent Bioanalyzer was required in order for samples to be sequenced.

		RNA Extraction	Bioanalyzer Results	
Lab ID	**Group**	**End volume (µl)**	**RIN**	**RNA concentration (ng/µl)**
SR081*	Control (TE)	80	8.7	20
SR038	Control (TE)	80	8.8	36
SR075	Control (TE)	80	7.1	13
SR146	Control (TE)	80	8.9	57
SR164	Control (TE)	80	9	64
SR072	Control (TE)	80	9	32
SR170	Control (TE)	80	9	28
SR048	Control (TE)	80	9.2	99
SR065	Control (TE)	80	9.1	98

SR066	Control (TE)	80	9	90
SR015	Control (TE)	80	9.3	20
SR016	Control (TE)	80	9.3	8
SR052	Control (TE)	80	9.3	145
SR001	Control (TE)	80	9.3	71
SR105	Control (TE)	80	9.3	124
SR096	Control (TE)	80	8.8	54
SR166	Control (TE)	40	8.9	197
SR214	Control (TE)	40	9.2	195
SR230	Control (TE)	40	8.9	138
SR279	Control (TE)	40	9.4	15
SR082	Control (TE)	40	9.6	47
SR109	Control (TE)	40	9.6	96
SR080	Control (TE)	40	9.3	73
SR013	Control (TE)	80	9.6	53
SR098	Control (TE)	80	7.8	30
SR055	Control (TE)	80	9.6	119
SR006	Control (TE)	80	9.3	37
SR119	Control (TE)	40	9.9	115
SR092	Control (TE)	40	8	243
SR089	Control (TE)	20	9.3	65
SR176	PTSD patient	80	8.8	77
SR148	PTSD patient	40	8.4	23
SR156	PTSD patient	40	8.8	60
SR158	PTSD patient	40	9.1	249
SR177	PTSD patient	40	9	125
SR150	PTSD patient	40	9	130
SR187	PTSD patient	40	8.9	136
SR140	PTSD patient	40	9	137
SR139	PTSD patient	40	9.2	151
SR190	PTSD patient	40	8.8	72
SR193	PTSD patient	40	8.4	155
SR186	PTSD patient	40	8.6	92
SR113	PTSD patient	40	9.1	72
SR058	PTSD patient	40	9.1	100
SR123*	PTSD patient	40	9.2	266
SR132	PTSD patient	40	9.2	179
SR135	PTSD patient	40	9.2	182
SR077	PTSD patient	40	9	117
SR019	PTSD patient	40	9.1	112
SR209	PTSD patient	40	9.2	184

PTSD – Post-traumatic stress disorder; TE - trauma-exposed; RIN - RNA integrity number

**Samples not sequenced due to inadequate library yield.*

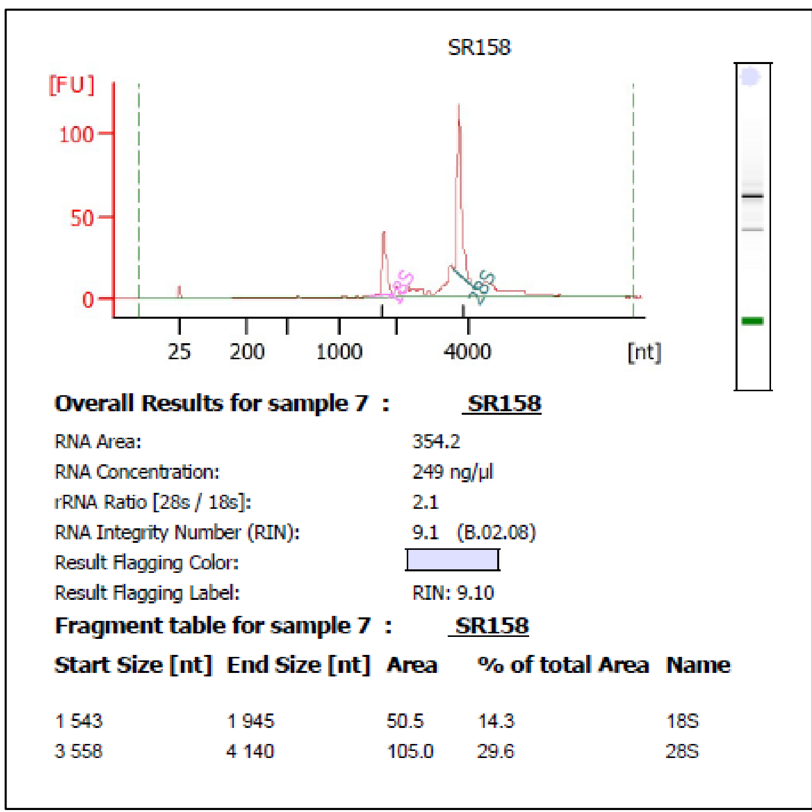

Figure 3.1: Representative Agilent Bioanalyzer result readout of an extracted RNA sample. Indicates the quality and concentration (ng/µl) of extracted tRNA with a RIN value ≥ 7 representing intact RNA of high quality adequate for RNA-sequencing analysis.

3.3 RNA sequencing

Of the 50 samples selected for sequencing, two samples (Patient SR123 and Control (TE) SR081) yielded inadequate sequencing libraries and were excluded. These samples had lower RNA concentrations (SR123 = 101.3 ng and SR081=169.6 ng) according to the Bioanalyzer results of the sequencing company, NXT-Dx (Ghent, Belgium). The remaining 48 samples were sequenced at a depth of 50 millions, paired end reads per sample with a read length of 50 base pairs using the Illumina HiSeq 4000 as sequencing platform. Whole transcriptome sequencing generated roughly 80GB of pooled raw RNA-Seq reads, in compressed FASTQ file format.

Using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), the base quality of the data indicated Phred scores (Q) of above 30, representing the high quality of the sequenced reads (Figure 3.2.a). Moreover, the sequence quality score report indicated a high sequencing quality distributed over all sequences (Figure 3.2.b).

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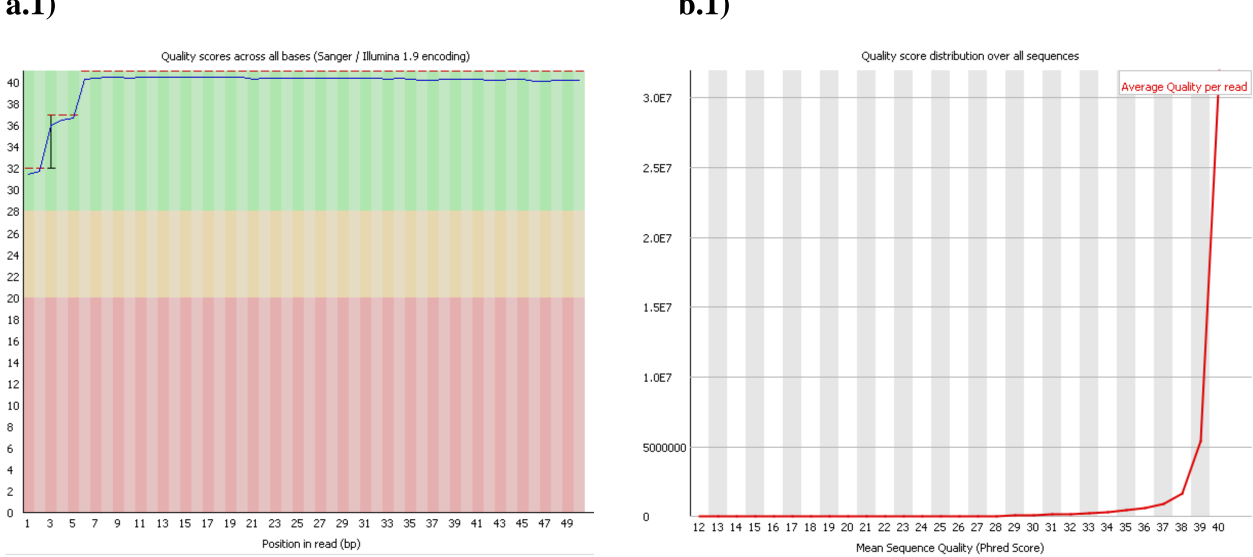
Example of forward read quality results as indicated by FASTQC:

a.1) **b.1)**



Example of reverse read quality results as indicated by FASTQC:

a.2) **b.2)**

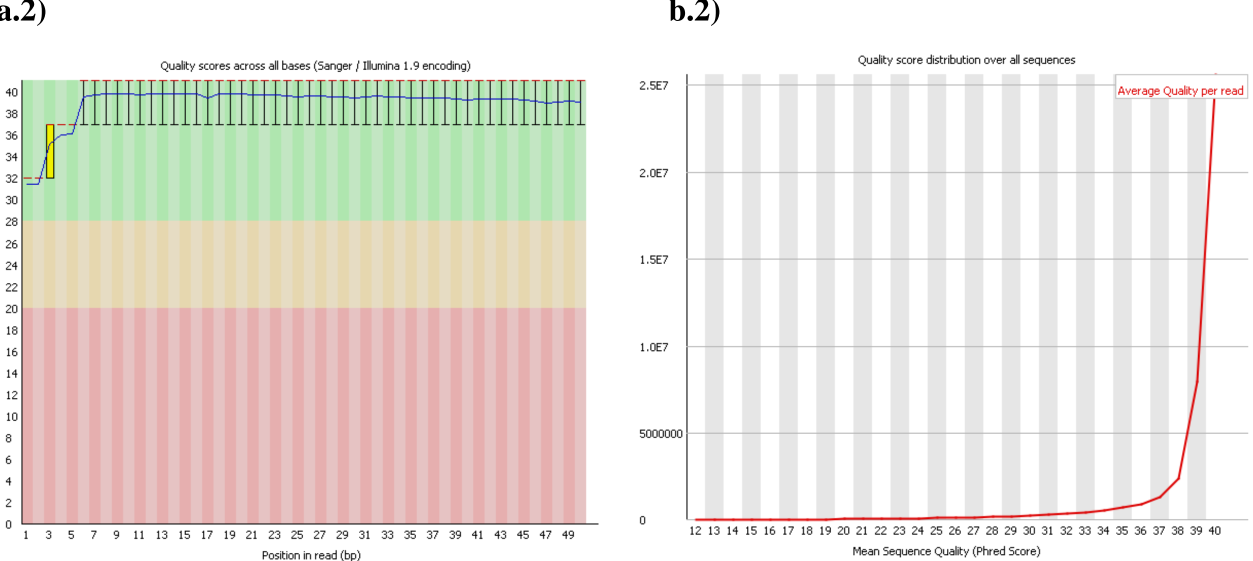


Figure 3.2: Phred (Q) scores of forward and reverse reads as indicated by FASTQC tool. a.1 & a.2) Indicates the base sequence quality where the x-axis represents the base pair position in the 50 bp read. The y-axis indicates the Phred quality score at each position with a Phred score of above 30 indicating a 1 in 1000 chance of a base being called wrong. The quality of the sequenced reads **b.1 & b.2)** indicates the average sequencing quality scores distributed over all sequences (used to identify poor quality reads within the entire sequencing reaction). The x-axis consists of the mean sequencing quality (Phred score) with the y-axis indicating read amounts.

3.4 Differential expression analysis using DESEQ2

From the differentially expressed gene list generated by DESEQ2 (https://bioconductor.org/packages/DESeq2), using an adjusted p-value of less than 0.05, identified a total of 556 significantly DEGs were identified in 48 samples. A Bland–Altman plot (MA plot) was generated by DESEQ2 indicating the log2 fold change of a given variable over the mean of normalized counts. By transforming the normalized counts data onto M (ratio) and A (mean average) scale, the MA plot provides a visual representation of the genomic data and therefore allows for a global view of all 556 DEGs between the case-control groups (Figure 3.3).

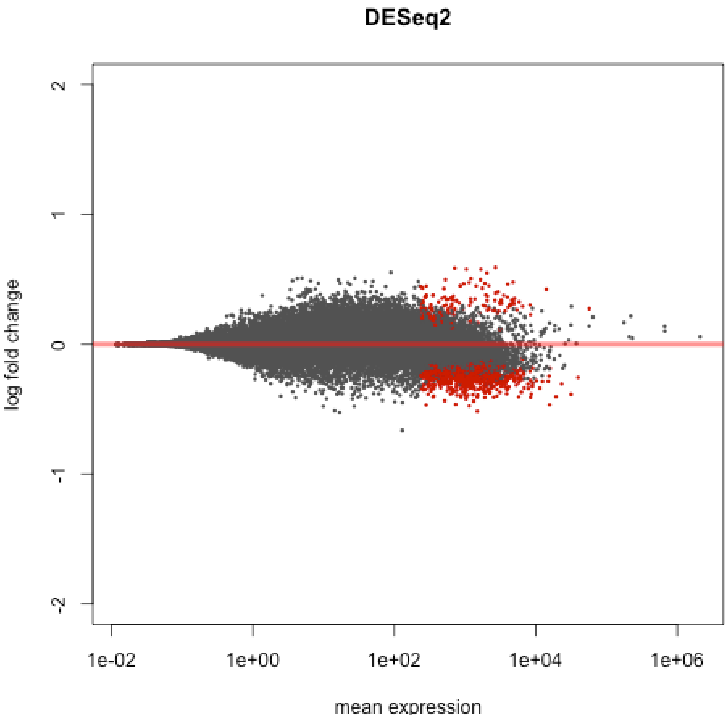


Figure 3.3: MA plot generated by DESEQ2. The MA plot indicates differential expression between 19 PTSD patients and 29 trauma-exposed controls. The x-axis represents the mean expression (normalised gene counts) with the y-axis representing the log2 fold change (normalised gene counts). Red coloured points represents gene sets differentially expressed at an adjusted p-value of less than 0.05. The upregulated gene sets was filtered at a log fold change of ≥ 0.25 and the downregulated genes set was filtered at a log fold change of ≤ -0.25, indicating highly DEGs.

Using the adjusted p-value of 0.05 and fold change of ≥ 0.25, 66 upregulated genes were identified. Similarly, an adjusted p-value of 0.05 with fold change of ≤ -0.25, resulted in the identification of 276 downregulated genes. Ideally, a fold change of less than -1 or higher than 1 would be selected as additional filtering requirement, however a lower fold change was selected due to the lack of differentiation within the given samples at this value.

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3.5 Using BioOntological Relationship Graph Database to identify gene-disease links

To further prioritize our set of 556 DEGs (66 up and 276 downregulated), anxiety-specific BORG database was used. Providing official gene symbols for each gene in the differentially expressed sets, the BORG database allowed for the identification of direct or transitive links between a gene and its function, phenotype and/or pathways that are also important features associated with the disease of interest.

Using Anxiety-specific BORG, potential links between the differentially expressed genes in PTSD compared to trauma-exposed controls, indicated that 196/556 DEGs (35% of the genes), link to anxiety, and by extension, to PTSD. Example of terms selected to transitively link a differentially expressed gene to its known functions, phenotypes and/or pathways included; "behavioral fear response" (GO), "fear-induced aggressive behavior" (GO), "abnormal fear/anxiety-related behavior" (HPO and MPO), "dopamine receptor binding" (GO), and "cortisol signaling pathway" (PW) among others, facilitating the discovery of biologically plausible genes, which might have been otherwise missed.

Of the 66 upregulated genes, 21 genes were associated to anxiety disorder. Of the 276 downregulated genes, 175 genes were associated to anxiety disorder using the BORG semantic database.

3.5.1 Upregulated genes associated with anxiety disorder using BORG semantic database

Of the 66 overexpressed genes, 21 genes were transitively associated with anxiety disorder based on their known functions, phenotypes or their role in a pathways important in anxiety and by extension in PTSD, further prioritizing our candidate gene list. GO terms such as "positive regulation of cytokine production" (GO:0001819), "learning and memory" (GO:0007611) and "calcium-mediated signalling" (GO:0019722) were functions associated with several genes and thus transitively linking the gene to the disease. Furthermore, "behavioural abnormality" (HP:0000708) and "stereotypic behavior" (HP:0000733) were the HPO terms associated with several of our potential candidate genes, linking them to PTSD. Additionally, "increased anxiety-related response" (MP:0001363), "abnormal cued conditioning behavior" (MP:0001454) and "decreased anxiety-related response" (MP:0001364) were phenotypes observed based on knock-out models (MPO) associated with some of the genes of interest and "neurotrophic factor signaling pathway" (PW:0000571), "long-term depression" (PW:0000061), "altered Reelin signaling pathway" (PW:0000390) and "glutamate signaling pathway" (PW:0000844) were associated pathways based on PW, transitively linked several genes to anxiety disorder.

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3.5.2 Downregulated genes associated with anxiety disorder using BORG semantic database

In the 175 downregulated genes, several GO terms, phenotypes observed in in both humans and knock-out models and pathways, were associated with each gene transitively linking them to the disease. “Positive regulation of cytokine production” (GO:0001819), “learning and memory” (GO:0007611) and “calcium-mediated signaling” (GO:0019722) were functions associated with some of the genes of interest linking them to anxiety disorder. Most frequent human phenotypes associated with several genes include “stereotypic behavior” (HP:0000733), “behavioral abnormality” (HP:0000708) and “anxiety” (HP:0000739). Whilst terms such as “increased thigmotaxis” (MP:0002797), “abnormal contextual conditioning behavior” (MP:0001469) and “increased anxiety related response” (MP:0001363) were the most common MPO terms associated with several genes in our set. Pathways such as “neurotrophic factor signaling pathway” (PW:0000571), “long-term depression” (PW:0000061) and “cortisol signaling pathway” (PW:0000569) were associated with some of the downregulated genes. Using the semantic model of the disease we were able to further prioritize the list of downregulated genes to those having key features of the disease based on known functions, phenotypes and associated pathways.

3.6 Gene set enrichment analysis through Enrichr

A total of 556 DEGs, consisting of up and down regulated gene sets, were submitted to Enrichr (http://amp.pharm.mssm.edu/Enrichr) using a crisp dataset containing official Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) gene symbols for humans. Gene set enrichment analysis produces a set of gene functions and pathways statistically overrepresented in a given set of genes, while our ontology driven candidate gene prioritization approach provides known functions, pathways and phenotypes, which are also hallmark of the disease, associated with each gene. For this reason and to reduce bias, differentially expressed set of genes identified prior to our BORG prioritization was used to carry out this analysis and to identify collective gene functions and pathways associated with a set of DEG rather than a direct or transitive link of gene-to-disease.

The combined score approach (computed by multiplying the log p-value from the Fisher exact test to the z-score of expected rank deviation) was selected to investigate GO terms, pathways (KEGG/WikiPath) and diseases that were enriched within the gene set. We report only on the top five functions, pathways and diseases due to more relevant terms being ranked higher based on the highest combined scores (Table 3.3).

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Biological processes enriched for the set of DEGs involved "protein localization to organelle" (GO:0033365), "establishment of protein localization to organelle" (GO:0072594), "gene expression" (GO:0010467), "single-organism cellular localization" (GO:1902580) and "viral transcription" (GO:0019083).

The highest ranked biological process GO term, "protein localization to organelle" (GO:0033365), consisted of 70 significantly enriched gene sets of which, 38 were upregulated (Table 3.3 upregulated gene set HGNC symbols indicated in red) and 32 downregulated (Table 3.3 downregulated gene set HGNC symbols indicated in black) at a combined score of 126.88 indicating the level of significance of the GO term. This was followed by the GO term "establishment of protein localization to organelle" (GO:0072594), leading to the enrichment of 61 of the 556 gene set of which, 37 were upregulated and 24 downregulated with a combined score of 122.89. The remaining GO terms "gene expression" (GO:0010467), "single-organism cellular localization" (GO:1902580) and "viral transcription" (GO:0019083) consisted of 85 (40 up and 45 downregulated), 73 (37 up and 36 downregulated) and 36 (all upregulated) overlapping gene sets for each respective GO term.

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Table 3.3: Top five significantly enriched biological process gene ontology (GO) terms based on a gene set of 556 DEGs. Gene names in red indicate upregulated genes within the gene set whilst black gene names indicate downregulated gene sets between PTSD patients and trauma-exposed controls. The number of significantly enriched gene sets are also indicated followed by the number of up and downregulated gene sets in brackets.

Name	p-value	Adjusted p-value	Z-score	Combined score	Number of genes (upregulated: downregulated)	Genes
Protein localization to organelle (GO:0033365)	2.22E-27	4.81E-24	-2.36	126.88	70 (38:32)	*RB1, RPL5, RPL30, RPL32, RPL31, LRRK2, RPL34, RPLP0, SRPR, ADAR, RPL9, RPL6, SYNE2, PPP3CA, RPS14, RPL35, RPL37, RPS11, KPNA3, RPL39, RPS13, RAB8B, RPS12, PDIA3, HSP90AA1, RPS7, RPL21, RPS8, RPS5, F2R, RPS6, RPL13A, RPS3A, PLRG1, YWHAZ, ACAP2, TXNIP, RPL24, RPL27, PHIP, RPL26, MAPRE1, KPNB1, ARF6, RPN2, RPL11, PIK3R1, FYB, RPS15A, TNKS2, SRP72, TPR, RPS3, RPL15, HSPA9, RANBP2, FIS1, NUP155, HSPA4, STAT3, RPL35A, DNAJA1, RPS29, RPL27A, RPS20, CALR, FAU, LAMTOR4, RPS21, RPS24*
Establishment of protein localization to organelle (GO:0072594)	2.89E-27	4.81E-24	-2.29	122.89	61(37:24)	*RPL5, RPL30, RPL32, RPL31, RPL34, SRPR, RPLP0, ADAR, RPL9, RPL6, PPP3CA, RPS14, LAMP1, RPL35, RPL37, RPS11, KPNA3, RPL39, RPS13, RPS12, RAB8B, PDIA3, HSP90AA1, RPS7, RPL21, RPS8, RPS5, F2R, RPS6, RPL13A, RPS3A, YWHAZ, RPL24, TXNIP, RPL27, PHIP, RPL26, KPNB1, RPN2, RPL11, PIK3R1, FYB, RPS15A, SRP72, TPR, RPS3, RPL15, HSPA9, RANBP2, FIS1, NUP155, HSPA4, STAT3, RPL35A, RPS29, RPL27A, RAB3GAP2, RPS20, FAU, RPS21, RPS24*
Gene expression (GO:0010467)	3.80E-24	1.26E-21	-2.32	111.51	85 (40:45)	*RPL5, RPL30, CCNT1, RPL32, YWHAB, RPL31, RPL34, RPLP0, SRPR, HNRNPU, ADAR, HNRNPR, EPRS, RPL9, RPL6, NARS, EFTUD2, EEF1B2, RPS14, SNRPD2, RPL35, PSMD1, RPL37, TNPO1, RPS11, RPL39, RPS13, RPS12, UPF2, SUPT16H, WARS, PARP1, RPS7, RPL21, RPS8, RPS5, RPS6, RPL13A, RPS3A, SMC1A, YWHAZ, CDC40, EEF1A1, MED23, XRN1, RPL24, RPL27, RPL26, SF3B5, GTF2A1, SF3B2, SET, SF3B3, RPN2, DHX9, RPL11, RPS15A, SRP72, POLR2B, CSTF1, RPS3, IARS2, RPL15,*

						SF3B1, SF3A3, RNGTT, USP9X, CPSF2, RPL35A, SSRP1, SNW1, HNRNPK, CNOT1, RPS29, CNOT2, RPL27A, HNRNPF, HNRNPA2B1, RPS20, FAU, RPS21, EIF3A, TRIM33, RPS24, EIF4G1
Single-organism cellular localization (GO:1902580)	3.27E-24	1.21E-21	-2.31	111.09	73 (37:36)	*RPL5, RPL30, RPL32, RPL31, RPL34, LRRK2, SRPR, RPLP0, ADAR, RPL9, PIK3CG, RPL6, SYNE2, PPP3CA, RPS14, KIF5B, RPL35, ITGB7, RPL37, RPS11, KPNA3, RPL39, RPS13, RAB8B, RPS12, MEF2A, ACTR3, PDIA3, ACTR2, MEF2C, HSP90AA1, ITGA4, RPL21, ANXA2, RPS7, RPS8, RPS5, RPS6, F2R, RPL13A, RPS3A, YWHAZ, RPL24, TXNIP, RPL27, PHIP, RPL26, KPNB1, RPN2, DCTN1, RPL11, PIK3R1, FYB, RPS15A, SRP72, TPR, RPS3, RPL15, HSPA9, RANBP2, FIS1, ICMT, NUP155, HSPA4, DENND4C, STAT3, RPL35A, RPS29, RPL27A, RPS20, FAU, RPS21, RPS24*
Viral transcription (GO:0019083)	4.51E-25	4.71E-22	-2.1	103.19	36 (36:0)	*RPL5, RPL30, RPL32, RPL31, RPL34, RPLP0, RPL11, RPL9, RPL6, RPS14, RPS15A, RPS3, RPL35, RPL15, RPL37, RPS11, RPL39, RPS13, RPS12, RPS7, RPL21, RPS8, RPS5, RPS6, RPL35A, RPL13A, RPS3A, RPS29, RPL27A, RPL24, RPS20, RPL27, RPL26, FAU, RPS21, RPS24*

The top five molecular functions enriched for the set of 556 DEGs were based on the highest combined scores which ranks these functions according to significance. At a combined score of 74.87, 36 genes, all upregulated, were significantly enriched for the GO term; "structural constituent of ribosome" (GO:0003735). The remaining GO terms; "Transcription factor binding" (GO:0008134), "DNA-dependent ATPase activity" (GO:0008094), "ATP binding" - (GO:0005524) and "DNA helicase activity" (GO:0003678), showed similar combined scores ranging from 20.39-27.08, with the majority of genes downregulated (Table 3.4).

Table 3.4: Top five significantly enriched molecular function gene ontology (GO) terms using a gene set of 556 DEGs. Gene names in red indicate upregulated genes within the gene set whilst black gene names indicate downregulated gene sets between PTSD patients and trauma-exposed controls. The number of significantly enriched gene sets are also indicated followed by the number of up and downregulated gene sets in brackets.

Name	p-value	Adjusted p-value	Z-score	Combined score	Number of genes (upregulated : downregulated)	Genes
Structural constituent of ribosome (GO:0003735)	2.93E-17	1.90E-14	-2.37	74.87	36 (36:0)	*RPL5, RPL30, RPL32, RPL31, RPL34, RPL11, RPLP0, RPL9, RPL6, RPS14, RPS15A, RPS3, RPL35, RPL15, RPL37, RPS11, RPL39, RPS13, RPS12, RPS7, RPL21, RPS8, RPS5, RPS6, RPL35A, RPL13A, RPS3A, RPS29, RPL27A, RPL24, RPS20, RPL27, RPL26, FAU, RPS21, RPS24*
Transcription factor binding (GO:0008134)	3.83E-08	0.00001239	-2.4	27.08	42 (3:39)	*RB1, GTF2A1, DDX3X, DHX9, CHD4, AHR, STK4, ETS1, HIF1A, ACTB, MTDH, CAND1, IFI16, GNPTAB, SIN3A, RPS3, NBN, MTA2, MEF2A, MEF2C, USP7, PARP1, NCOA3, STAT3, ARNT, COMMD6, YWHAZ, SIRT1, NFKB1, REST, SNW1, CNOT1, IRF4, THRAP3, CNOT2, KAT6A, HNRNPF, BHLHE40, CTNNB1, TCF4, TP53, TPT1*
DNA-dependent ATPase activity (GO:0008094)	4.87E-07	0.0000827	-2.37	22.25	15 (0:15)	*TOP2B, XRCC6, DDX3X, RFC1, DHX9, XRCC5, CHD8, ATRX, RECQL, CHD4, SMARCA2, CHD1, RBBP4, G3BP1, NBN*
ATP binding (GO:0005524)	5.11E-07	0.0000827	-2.36	22.2	88 (1:88)	*TOP2B, HSP90AB1, DDX3X, DDX46, LRRK2, CHD8, RTCB, HNRNPU, CHD4, DDX60L, SMC6, EPRS, SMC3, CHD1, PIK3CG, ACTB, NARS, RPS6KA3, SLK, KIF5B, NMRK1, JAK1, ACTR3, ACTR2, DDX18, HSP90AA1, WARS, RFC1, CSNK2A1, SWAP70, ATRX, RECQL, ACSL5, ATP11C, ACSL4, ATP11B, ACSL3, BAZ1B, SMC1A, PKM, THRAP3, TCP1, MCM3, GART, TP53, ATP6V1A, VCP, PRKAA1, PXK, DHX8, ROCK1, DHX9, UBA6, VPS4B, PIK3R4, NOLC1, DDX21, STK4, UBE2J1, HSP90B1, HSPD1, HSPH1, BMS1, G3BP1, PGK1, STK38L, IARS2, CCT5, HSPA9, CDK17, XRCC6, MAP3K1, HSPA5, HSPA4, XRCC5, EIF2AK3, ATP2B4, EIF2AK4, ATP2B1, HIPK1, SMARCA2, CCT6A, DNAJA1, GLUD1, TAOK3, ETNK1, DNAJA2, UBA1*
DNA helicase activity (GO:0003678)	8.67E-07	0.0001121	-2.24	20.39	12 (0:12)	*XRCC6, DDX3X, XRCC5, DHX9, CHD8, ATRX, G3BP1, RECQL, MCM3, CHD4, NBN, CHD1*

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The "Ribosome" (hsa03010) pathway, enriched for 36 upregulated gene sets, was identified as the top Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway based on *Enrichr* (http://amp.pharm.mssm.edu/Enrichr). These genes correspond to biological process (Table 3.3) and molecular function (Table 3.4) GO terms; "viral transcription" (GO:0019083) and "structural constituent of ribosome" (GO:0003735) respectively. Other significantly enriched pathways included the "Spliceosome" pathway (hsa03040), enriched for 24 genes 20 of which were downregulated, "Protein processing in endoplasmic reticulum" (hsa04141), with 26 genes enriched, "viral carcinogenesis" (hsa05203), with 19 genes enriched and the 'HIF-1 signaling pathway' (hsa04066) with 12 genes significantly enriched in the pathway. (Table 3.5)

Table 3.5: Top five enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of based on a gene set of 556 DEGs. Gene names in red indicate upregulated genes within the gene set whilst black gene names indicate downregulated gene sets between PTSD patients and trauma-exposed controls. The number of significantly enriched gene sets are also indicated followed by the number of up and downregulated gene sets in brackets.

Name	p-value	Adjusted p-value	Z-score	Combined score	Number of genes (upregulated : downregulated)	Genes
Ribosome (hsa03010)	9.61E-17	2.30E-14	-1.75	54.84	36 (36:0)	*RPL5, RPL30, RPL32, RPL31, RPL34, RPL11, RPLP0, RPL9, RPL6, RPS14, RPS15A, RPS3, RPL35, RPL15, RPL37, RPS11, RPL39, RPS13, RPS12, RPL21, RPS7, RPS8, RPS5, RPS6, RPL13A, RPL35A, RPS3A, RPS29, RPL27A, RPL24, RPL27, RPS20, FAU, RPL26, RPS21, RPS24*
Spliceosome (hsa03040)	1.38E-08	0.000001654	-1.77	23.59	24 (2:22)	*SF3A3, SF3B5, SF3B2, AQR, PRPF38B, RBM25, SF3B3, DHX8, DDX46, HNRNPU, CDC5L, PRPF40A, PLRG1, WBP11, CRNKL1, CDC40, U2SURP, EFTUD2, HNRNPK, RBMXL1, SNW1, SNRPD2, SF3B1, RBM22*
Protein processing in endoplasmic reticulum (hsa04141)	5.39E-08	0.000004293	-1.77	21.83	26 (2:24)	*VCP, HSP90AB1, RPN2, SEL1L, CUL1, HSP90B1, UBE2J1, LMAN1, HSPH1, SEC23B, SEC31A, PDIA3, EDEM3, HSP90AA1, HSPA5, EIF2AK3, EIF2AK4, RBX1, PDIA4, DNAJA1, DNAJA2, CANX, STT3A, CALR, SEC24D, ATF4*
Viral carcinogenesis (hsa05203)	0.00159	0.08807	-1.91	4.63	19 (1:18)	*RB1, GTF2A1, USP7, DDX3X, YWHAB, STAT3, CHD4, PIK3R1, YWHAZ, NFKB1, PIK3CG, DDB1, HNRNPK, SNW1, PKM, TP53, YWHAG, ATF4, JAK1*
HIF-1 signaling pathway (hsa04066)	0.00212	0.08807	-1.74	4.22	12 (2:10)	*LDHA, RPS6, STAT3, PGK1, CYBB, ARNT, PIK3R1, ENO1, HIF1A, NFKB1, PIK3CG, RBX1*

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Of the 556 DEG set, 37 genes, 36 up and 1 downregulated gene were enriched for the "Cytoplasmic Ribosomal Proteins" (WP477) WikiPathway. Other pathways with enriched gene sets included genes involved in "mRNA Processing" (WP411), "Apoptosis-related network due to altered Notch3 in ovarian cancer" (WP2864), "EGF/EGFR Signaling Pathway" (WP437) and "BDNF signaling pathway" (WP2380) with the majority of genes in the pathways downregulated (Table 3.6).

Table 3.6: Top five enriched WikiPathways of the based on a gene set of 556 DEGs. Gene names in red indicate upregulated genes within the gene set whilst black gene names indicate downregulated gene sets between PTSD patients and trauma-exposed controls. The number of significantly enriched gene sets are also indicated followed by the number of up and downregulated gene sets in brackets.

Name	p-value	Adjusted p-value	Z-score	Combined score	Number of genes (upregulated : downregulated)	Genes
Cytoplasmic ribosomal proteins (WP477)	3.91E-20	6.02E-18	-1.98	78.6	37 (36:1)	*RPL5, RPL30, RPL32, RPL31, RPL34, RPL11, RPLP0, RPL9, RPL6, RPS6KA3, RPS14, RPS15A, RPS3, RPL35, RPL15, RPL37, RPS11, RPL39, RPS13, RPS12, RPL21, RPS7, RPS8, RPS5, RPS6, RPL13A, RPL35A, RPS3A, RPS29, RPL27A, RPL24, RPL27, RPS20, FAU, RPL26, RPS21, RPS24*
mRNA processing (WP411)	6.26E-06	0.0004816	-1.89	14.42	21 (2:16)	*SF3A3, SF3B5, SF3B2, SF3B3, RNGTT, DHX8, DHX9, NONO, CPSF2, HNRNPU, HNRNPR, PRPF40A, SMC1A, CDC40, EFTUD2, HNRNPK, SNRPD2, XRN2, HNRNPA2B1, CSTF1, SF3B1*
Apoptosis-related network due to altered Notch3 in ovarian cancer (WP2864)	0.002532	0.1114	-1.83	4.01	18 (2:16)	*VAV3, MAP3K1, YWHAB, STAT3, PIK3R1, PIK3CG, EEF1A1, RPS6KA3, PLSCR1, ITCH, SNRPD2, PRKAR1A, SIN3A, ABI1, RBBP7, MTA2, EPS15, JAK1*
EGF/EGFR signaling pathway (WP437)	0.005436	0.186	-1.87	3.15	17 (0:17)	*VAV3, MEF2A, USP8, MEF2C, MAP3K1, ROCK1, NCOA3, STAT3, PIK3R1, IQGAP1, RPS6KA3, PLSCR1, ITCH, ABI1, EPS15, ARF6, JAK1*
BDNF signaling pathway (WP2380)	0.008864	0.2306	-1.95	2.86	15 (1:14)	*VAV3, MEF2A, PRKAA1, MEF2C, MAP3K1, CSNK2A1, KIDINS220, RPS6, STAT3, PIK3R1, NFKB1, RPS6KA3, MARCKS, DPYSL2, CTNNB1*

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Of the top five OMIM diseases, only two gene sets ("Anemia" consisting of five upregulated gene set and "Mental retardation" consisting of six downregulated gene set), were significantly enriched for based on a p-value of less than 0.05. None of these OMIM diseases were identified as significant according to the adjusted p-value (Table 3.7).

Table 3.7: Top five enriched Online Mendelian Inheritance in Man (OMIM) diseases based on a gene set of 556 DEGs. Gene names in red indicate upregulated genes within the gene set whilst black gene names indicate downregulated gene sets between PTSD patients and trauma-exposed controls. The number of significantly enriched gene sets are also indicated followed by the number of up and downregulated gene sets in brackets.

Name	p-value	Adjusted p-value	Z-score	Combined score	Number of genes(upregulated : downregulated)	Genes
Anemia	0.01007*	0.272	-1.73	2.26	5 (5: 0)	*RPL5, RPS7, RPL11, RPL35A, RPS24*
Mental retardation	0.03301*	0.3824	-1.76	1.69	6 (0: 6)	*RPS6KA3, SLC9A6, ATP6AP2, ATRX, ACSL4, ARHGEF6*
Thyroid carcinoma	0.05668	0.3824	-0.82	0.78	2 (0: 2)	*PRKAR1A, TRIM33*
Parkinson disease	0.08583	0.3824	-0.61	0.59	2 (0: 2)	*LRRK2, GIGYF2*
Immunodeficiency	0.1254	0.3824	-0.57	0.54	2 (0: 2)	*PTPRC, IL2RG*

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3.7 Identifying gene set co-expression using COXPRESdb

The 196 biologically-relevant gene sets identified by the anxiety BORG were submitted for co-expression analysis to identify genes that are functionally related or members of the same pathway. This allowed for the identification of dysregulated molecular pathways possibly involved in the formation or maintenance of PTSD.

The 196 official HGNC gene symbols were converted, using the ID converter system (http://biodb.jp/idc.cgi), to their respective Entrez Gene IDs before being submitted to the database COXPRESdb (http://coxpresdb.jp), as the required input format. The EdgeAnnotation function of COXPRESdb was selected to extract co-expression data for all 196 differentially expressed query genes. The "all possible pairs in the query genes" option was selected for our query gene set, in the EdgeAnnotation function, which returned a set of co-expressed genes. The co-expression data reported on conserved co-expression, correlation, as well as the mutual rank (MR) value between two query genes (the MR is a geometric, averaged correlation rank calculated between the two directional ranks (see Chapter 2 Section 2.10)). A stringent cut-off (MR < 5) was selected, leaving 45 co-expression pairs (from a list of over 2000 gene pairs) that were highly similar in terms of co-expression and would therefore most likely be functionally related or members of the same pathway (Table 3.8). Correlation of the expression pattern between two example genes (*HSPH1* and *HSP90AA1*) are illustrated in Figure 3.4. The co-expression data was subsequently ordered by gene name in Excel, identifying a total of 64 genes (MR < 5), which were highly co-expressed with four upregulated and 60 downregulated genes.

Table 3.8: COXPRESdb, EdgeAnnotation results of the co-expression (MR < 5) between 196 biologically relevant genes (up- and downregulated) identified through BORG analysis.

Gene1	Entrez Gene ID	Gene2	Entrez Gene ID	Mutual Rank	Correlation	Conserved co-expression
HSPH1	10808	*HSP90AA1*	3320	1	0.59	Yes
HSPA5	3309	*HSP90B1*	7184	1	0.63	Yes
GBP1	2633	*GBP2*	2634	1	0.64	
DDX3X	1654	*G3BP2*	9908	1	0.68	
SMC3	9126	*BCLAF1*	9774	1	0.65	Yes
HNRNPK	3190	*HNRNPU*	3192	1.4	0.62	
ENO1	2023	*PGK1*	5230	1.4	0.71	Yes
ACTR2	10097	*DDX3X*	1654	1.4	0.67	
COPA	1314	*DHX9*	1660	1.4	0.67	
STIP1	10963	*HSPA4*	3308	1.4	0.66	Yes
LDHA	3939	*PGK1*	5230	1.7	0.63	Yes
STIP1	10963	*HSP90AB1*	3326	2	0.71	Yes
STAG2	10735	*ZNF148*	7707	2	0.54	
RPL6	6128	*RPS6*	6194	2	0.71	
NCL	4691	*NOLC1*	9221	2.2	0.58	
CHD4	1108	*THRAP3*	9967	2.5	0.6	
DCTN1	1639	*TLN1*	7094	2.5	0.55	
POLR2B	5431	*UBA6*	55236	2.5	0.53	
STAG2	10735	*XIAP*	331	2.5	0.5	
DDX21	9188	*NOLC1*	9221	2.8	0.6	
MEF2C	4208	*TCF4*	6925	2.8	0.37	Yes
DHX9	1660	*EPRS*	2058	2.8	0.63	
PDIA3	2923	*HSPA5*	3309	3	0.55	
DHX9	1660	*BCLAF1*	9774	3.2	0.65	Yes
EPRS	2058	*VPS35*	55737	3.2	0.59	
CLTC	1213	*COPB2*	9276	3.2	0.46	Yes
ANXA2	302	*ANXA5*	308	3.7	0.42	Yes
ZBTB33	10009	*STAG2*	10735	3.7	0.45	
KLRD1	3824	*PRF1*	5551	3.7	0.64	Yes
TPR	7175	*SMC3*	9126	4	0.63	
EIF4G1	1981	*VCP*	7415	4	0.52	
HSPA4	3308	*HSPA9*	3313	4.1	0.6	Yes
ANXA2	302	*CAST*	831	4.1	0.42	
EIF4G1	1981	*IQGAP1*	8826	4.2	0.57	
ACTR2	10097	*G3BP2*	9908	4.2	0.63	
ATRX	546	*SMC3*	9126	4.2	0.64	
EZR	7430	*BCLAF1*	9774	4.5	0.55	
HSPH1	10808	*DNAJA1*	3301	4.6	0.48	Yes
STIP1	10963	*ENO1*	2023	4.6	0.66	
DHX9	1660	*VPS35*	55737	4.8	0.59	
ETS1	2113	*MSN*	4478	4.9	0.38	
COPB1	1315	*CTR9*	9646	4.9	0.49	
DNAJA1	3301	*HSP90AA1*	3320	4.9	0.49	Yes
EEF1B2	1933	*RPS3A*	6189	4.9	0.72	
SF3B2	10992	*SSRP1*	6749	4.9	0.55	

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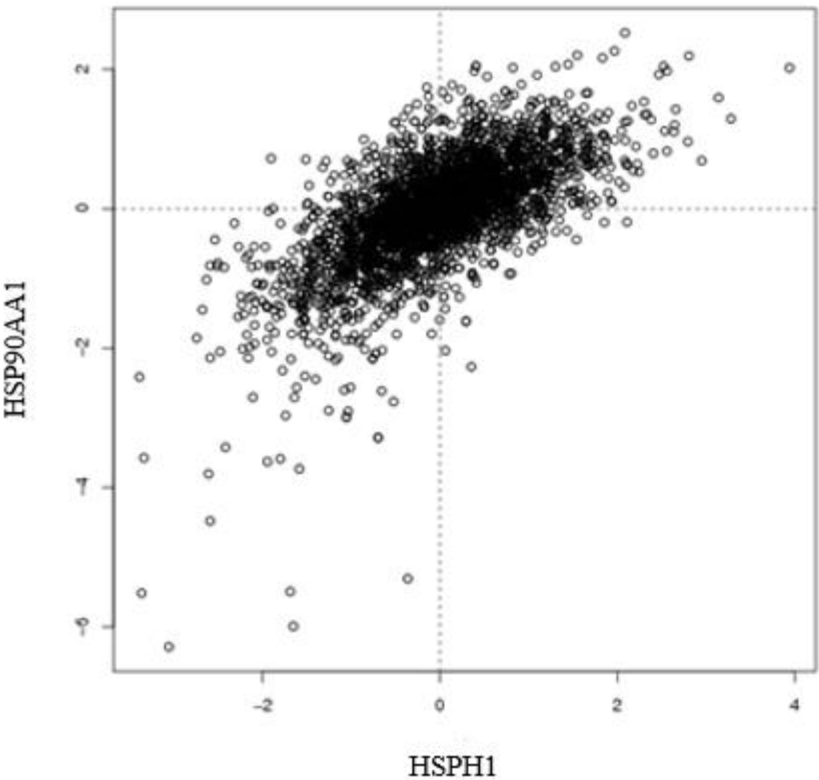

Figure 3.4: Example of gene co-expression correlation generated by COXPRESdb. Both x and y axes indicate relative gene expression values in base-2 logarithm against the averaged expression levels of each gene. The *HSPH1* relative gene expression on the x-axis is compared to *HSP90AA1* relative gene expression on the y-axis. Correlation of the expression pattern between these two genes indicate similar co-expression patterns suggesting genes are functionally related or likely members of the same pathway.

The 64 co-expressed genes, identified by the EdgeAnnotation function, were submitted to the NetworkDrawer function of COXPRESdb in order to draw a co-expressed gene network. Using the Entrez Gene IDs, all 64 genes were submitted, and the Graphviz display type option was selected. Ten co-expression networks were identified, one up- and nine downregulated. Figure 3.5 illustrates these co-expression networks based on the strength of co-expression between two genes through MR values. The strongest co-expressions are indicated by bold edges (MR < 5), followed by normal edges (MR < 30), whilst the weaker connections are illustrated by thin edges (MR ≥ 30). The light-grey nodes in Figure 3.5 show the 64 query input genes, with the dark-grey nodes indicating additional co-expressed genes added by the NetworkDrawer. Orange edges indicate conserved co-expression between species based on NCBI HomoloGene and COXPRESdb data with red dotted edges representing protein-protein interactions based on the Human Protein Reference Database.

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The network indicated KEGG pathways (obtained from KEGG database) through coloured dots in both query and additional nodes showing that seven genes (*HSP90AA1, HSP90AB1, XIAP, TPR, CTNNA1, ETS1* and *HSP90B1*) are involved in pathways identified in cancer (hsa05200) with another seven genes (*HSP90AA1, HSP90AB1, HSPA8, HSPA4, HSPA5, PDIA3* and *KLRD1*) implicated in antigen processing and presentation pathways (hsa04612). Other KEGG pathways of the co-expression network included ribosome (hsa03010), four genes (*RPS6, RPL6, RPL5* and *RPS3A*), glycolysis / gluconeogenesis (hsa00010), four genes (*ENO1, PGK1 LDHA* and *PGAM1*) and spliceosome (hsa03040), four genes (*SF3B2, HNRNPU, HNRNPK* and *HSPA8*) (Table 3.9).

3.7.1 Co-expressed upregulated gene sets at an MR value lower than five as identified by COXPRESdb

From the co-expression networks constructed by the NetworkDrawer tool of COXPRESdb, all four upregulated input query genes; *RPS6, RPL6, EEF1B2* and *RSP3A* (represented by light-grey nodes in Figure 3.5) co-expressed together along with one additional gene, *RPL5* (represented by dark-grey nodes in Figure 3.5) added by the NetworkDrawer (Figure 3.5). The co-expressed list of upregulated genes was selected for further gene expression analysis in tissues, using GTEx database (http://gtexportal.org).

3.7.2 Co-expressed downregulated gene sets at an MR value lower than five as identified by COXPRESdb

The remaining 60 genes that co-expressed with each other were downregulated genes in our dataset. Two genes (*TLN1* and *DCTN1*) did not show co-expression on the NetworkDrawer, leaving 58 genes from which co-expressions networks were drawn. A total of ten genes co-expressed only with one other gene (5 co-expression pathways), 12 genes co-expressed with only two or three other genes (3 co-expression pathways) and 36 genes co-expressed as part of a larger network. The network of 36 genes was selected for tissue specific gene expression analysis using GTEx (http://gtexportal.org) as such a network would most likely have a biological effect when dysregulated.

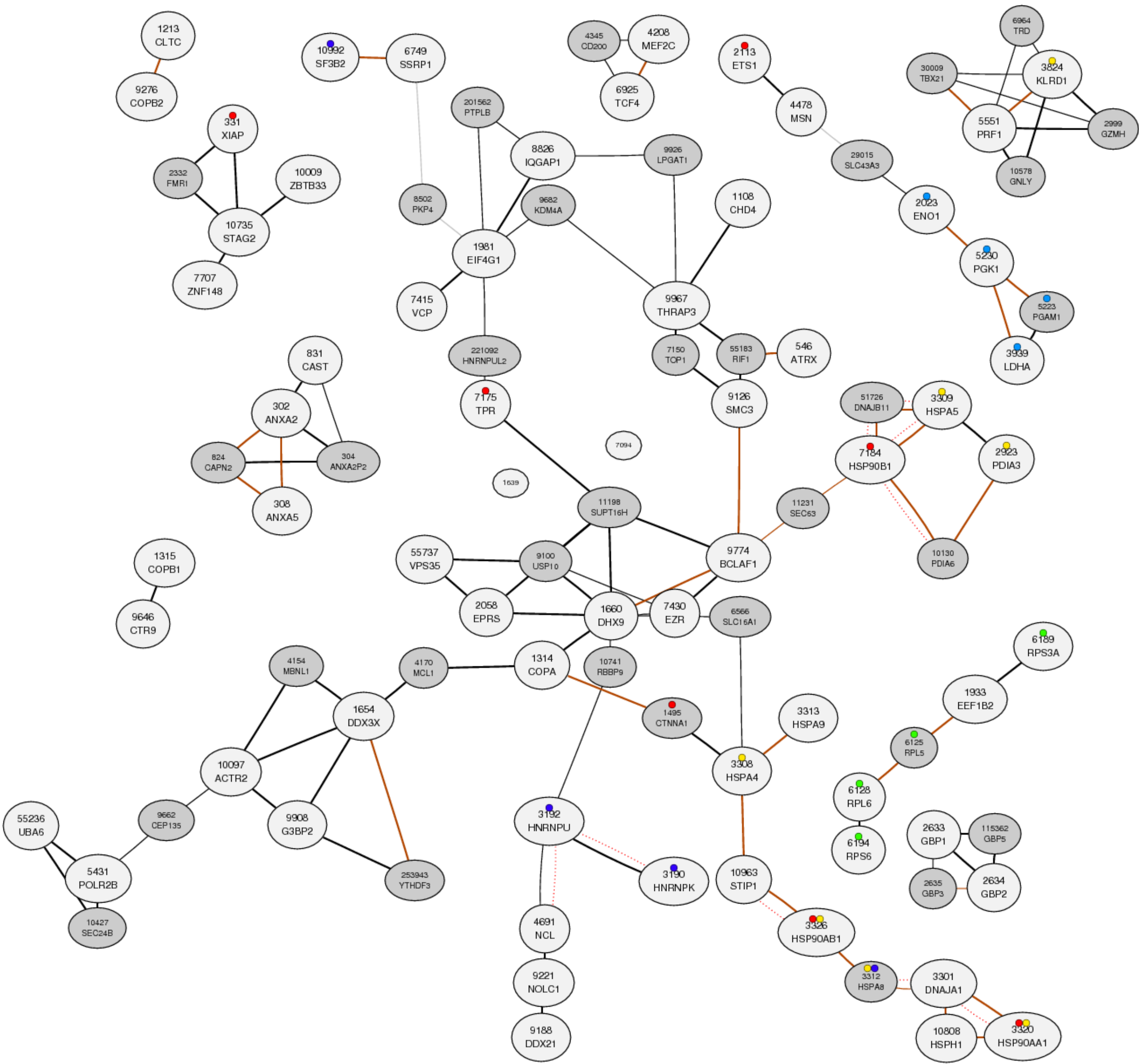

Figure 3.5: Co-expression network generated by the NetworkDrawer tool in COXPRESdb illustrates the co-expression of genes in terms of mutual rank (MR) values with the strength of co-expression shown as bold edges (MR < 5), normal (MR < 30) and weak (MR ≥ 30). Orange edges indicate conserved co-expression (based on NCBI HomoloGene and COXPRESdb) with red dotted edges indicating protein-protein interactions (based on the Human Protein Reference Database). Light grey nodes show query genes whilst dark grey nodes indicate additional genes connected to the network and added by the NetworkDrawer. Coloured dots in nodes indicate KEGG pathways (See Table 3.9 below).

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Table 3.9: KEGG pathways linked to NetworkDrawer co-expression map in Figure 3.5 indicates the top KEGG pathways linked to several genes in the co-expression network generated through COXPRESdb.

KEGG ID	Term	Number of genes	KEGG map link
hsa05200	Pathways in cancer	7	🔴
hsa04612	Antigen processing and presentation	7	🟡
hsa03010	Ribosome	4	🟢
hsa00010	Glycolysis / Gluconeogenesis	4	🔵
hsa03040	Spliceosome	4	⚫

A subset of the most powerful candidate genes were selected for further investigation based on anxiety BORG and COXPRESdb co-expression analysis.

3.8 Tissue expression identification using the GTEx portal

To identify whether the co-expressed genes were also expressed in different brain tissues, the Genotype-Tissue Expression (GTEx) (http://gtexportal.org) portal was used. The database provides insight on healthy human gene expression patterns by reporting on Reads Per Kilobase of transcript per Million mapped reads (RPKM) expression values for several tissues.

The official HGNC gene symbols of the co-expression network of four upregulated and the large downregulated gene network of 36 genes, were individually searched for in the GTEx portal. The gene expression data for each gene was filtered by tissue, selecting 14 of the 53 tissues available in the database. Selected genes included all 13 brain tissues; amygdala, anterior cingulate cortex, caudate, cerebellar hemisphere, cerebellum, cortex, frontal cortex, hippocampus, hypothalamus, nucleus accumbens, putamen, spinal cord and substantia nigra as well as whole blood (which was selected to estimate the differences between expression levels for each gene).

The median RPKM values of the 14 tissues were reported for the four upregulated (Table 3.10) and 36 downregulated (Table 3.11) gene sets. Tissue expression of an additional downregulated gene, Myeloid Cell Leukemia 1 (*MCL1*), (gene not part of the input genes but an additional gene added by the NetworkDrawer) was also reported due to the gene being highly connected, co-expressing with several genes in the large downregulated network identified by COXPRESdb.

Of the upregulated gene set, all four genes were highly expressed within the brain tissues compared to whole blood tissue, with Eukaryotic Translation Elongation Factor 1 beta 2 (*EEF1B2*) showing the

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lowest whole blood expression levels (median RPKM of 26.715) compared to the three other upregulated genes. Ribosomal Protein S6 (*RPS6*) brain expression was reported to be the highest within the amygdala, spinal cord and substantia nigra with a median RPKM of 232.195, 332.132 and 264.603 respectively (Table 3.10).

The downregulated gene set of 36 genes and the additional gene *MCL1*, were all expressed within brain tissues with two genes, IQ Motif Containing GTPase Activating Protein 1 (*IQGAP1*) and DEAD-Box Helicase 21 (*DDX21*), showing low brain expression median RPKM levels compared to whole blood tissue expression (Table 3.11).

The tissue expression analysis by GTEx aided in the selection of ten genes which are discussed within Chapter 4 as these genes were identified as potentially relevant links to anxiety disorder and by extension to PTSD based on multiple analyses. Selected genes included the four co-expressed, upregulated gene set: *RPS6, RPL6, RPS3A* and *EEF1B2*, as well as six highly connected, downregulated, co-expressed gene set: *EIF4G1, HSPA4, DHX9, BCLAF1, THRAP3* and *MCL1*.

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Table 3.10: Tissue expression identification through GTEx portal consisting of RPKM median gene expression levels of healthy individuals from brain and whole blood tissue. Compares gene tissue expression of four upregulated genes identified by anxiety BORG and co-expressed analysis in order to identify DEG sets between PTSD patients and Trauma-exposed control individuals.

	RPKM median GTEx													
HGNC gene symbol	**Amygdala (n=72)**	**Anterior cingulate cortex (n=84)**	**Caudate (Basal ganglia) (n=117)**	**Cerebellar Hemisphere (n=105)**	**Cerebellum (n=125)**	**Cortex (n=114)**	**Frontal cortex (n=108)**	**Hippocampus (n=93)**	**Hypothalamus (n=96)**	**Nucleus accumbens (basal ganglia) (n=113)**	**Putamen (Basal ganglia) (n=97)**	**Spinal cord (cervical c-1) (n=71)**	**Substantia nigra (n=63)**	**Whole Blood (n=393)**
RPL6	96.44	75.115	74.367	106.526	95.989	69.837	72.825	76.713	90.62	91.275	71.305	98.069	93.146	53.465
EEF1B2	87.668	64.826	72.861	70.741	66.411	61.966	64.594	79.619	88.035	98.321	73.197	111.582	108.078	26.715
RPS6	232.195	144.485	169.872	194.419	174.948	129.375	137.308	190.053	208.618	195.219	172.404	332.132	264.603	161.553
RPS3A	53.218	40.797	38.465	67.921	60.146	39.521	42.094	43.261	49.775	55.335	35.02	61.394	58.277	21.979

Table 3.11: Tissue expression identification through GTEx portal consisting of RPKM median gene expression levels of healthy individuals from brain and whole blood tissue. Compares gene tissue expression 37 downregulated genes identified by anxiety BORG and co-expressed analysis in order to identify DEG sets between PTSD patients and Trauma-exposed control individuals.

	RPKM median GTEx													
HGNC gene symbol	**Amygdala (n=72)**	**Anterior cingulate cortex (n=84)**	**Caudate (Basal ganglia) (n=117)**	**Cerebellar Hemisphere (n=105)**	**Cerebellum (n=125)**	**Cortex (n=114)**	**Frontal cortex (n=108)**	**Hippocampus (n=93)**	**Hypothalamus (n=96)**	**Nucleus accumbens (basal ganglia) (n=113)**	**Putamen (Basal ganglia) (n=97)**	**Spinal cord (cervical c-1) (n=71)**	**Substantia nigra (n=63)**	**Whole Blood (n=393)**
SF3B2	28.996	30.233	36.974	52.21	51.629	35.293	33.663	29.681	32.354	36.589	32.877	30.288	30.874	36.511
SSRP1	16.417	18.301	19.485	36.86	36.888	25.22	23.044	17.723	21.355	20.527	16.647	17.083	17.023	11.127
VCP	22.492	27.123	24.764	39.261	37.118	34.039	35.842	24.386	34.074	28.049	21.76	32.027	28.297	29.578
EIF4G1	20.082	21.355	19.861	25.765	29.37	26.358	23.169	20.462	24.619	20.197	19.482	20.96	23.418	11.464
IQGAP1	6.411	4.021	7.169	4.562	5.003	5.26	4.506	8.444	6.035	5.122	6.068	3.013	9.685	53.302
CHD4	15.849	13.927	20.817	30.219	28.141	16.944	15.774	15.748	17.221	18.927	18.81	19.244	19.403	14.093
THRAP3	13.037	13.835	14.555	28.433	24.777	14.686	15.835	13.459	15.479	15.228	11.68	13.348	12.805	19.817
ATRX	3.83	4.594	4.111	9.412	8.405	4.441	5.31	3.906	4.605	4.43	3.359	3.579	3.445	1.604
SMC3	6.271	5.066	6.977	17.039	14.354	5.337	5.347	6.088	6.719	7.284	6.134	8.338	6.502	3.754
BCLAF1	8.925	9.918	10.421	24.933	21.482	10.564	12.28	9.458	12.076	11.222	8.939	10.916	9.211	6.733

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HSP90B1	66.075	67.142	85.943	62.416	49.424	55.486	83.041	68.446	95.666	91.667	74.989	114.888	92.151	43.643
HSPA5	31.5	34.356	45.732	40.315	31.3	28.585	40.17	32.906	54.755	48.078	37.535	53.177	37.9	65.979
PDIA3	22.688	20.169	25.122	27.117	22.301	18.593	24.56	22.767	31.65	27.957	23.147	35.919	28.955	30.508
TPR	5.189	4.722	5.67	11.839	11.903	5.771	5.477	5.078	5.511	6.009	5.139	5.827	5.583	6.737
EPRS	9.294	10.457	10.815	19.389	17.152	12.45	12.85	9.45	11.817	12.2	9.457	13.272	11.027	4.697
VPS35	7.658	9.012	9.037	11.988	9.659	10.174	15.189	7.953	12.559	9.848	7.149	8.257	8.836	11.004
EZR	37.688	37.504	48.88	2.341	3.47	32.28	29.02	23.787	23.023	36.618	35.653	16.856	21.75	26.071
DHX9	12.883	13.701	16.209	31.739	25.669	14.38	16.048	13.032	15.367	16.299	13.108	15.398	13.16	11.584
COPA	20.645	24.118	25.9	44.022	38.161	29.363	33.315	23.282	28.244	26.606	20.71	32.581	26.418	27.485
DDX3X	15.667	16.77	17.892	28.217	25.781	19.413	20.018	16.166	18.599	17.469	14.312	21.114	17.664	26.222
ACTR2	30.416	38	32.891	41.084	28.742	32.935	53.94	30.703	32.868	34.76	28.278	37.41	31.701	58.475
G3BP2	11.611	17.041	12.299	32.166	21.043	15.694	27.216	11.825	19.686	16.948	9.82	15.527	12.8	6.335
POLR2B	8.149	10.124	10.526	28.063	23.228	12.727	14.037	8.934	10.915	10.151	8.685	8.943	8.627	8.728
UBA6	2.526	2.385	2.431	5.344	5.571	2.815	2.716	2.667	3.254	2.552	2.051	4.063	2.984	1.617
HNRNPU	29.032	26.944	31.437	58.641	49.86	27.055	30.134	28.524	32.037	34.625	27.444	33.509	29.337	24.807
HNRNPK	68.927	68.37	71.715	114.988	93.685	65.013	78.91	69.136	82.476	77.512	62.147	85.116	70.327	101.015
NCL	46.088	41.229	47.767	78.53	62.623	41.865	48.177	47.213	50.382	49.547	46.072	64.625	51.155	19.862
NOLC1	13.373	14.591	14.269	30.074	23.671	17.315	19.169	12.742	15.249	14.668	12.15	13.836	12.903	3.746
DDX21	4.101	3.596	4.188	5.058	4.615	3.673	4.078	4.17	4.829	3.988	3.599	6.568	4.97	6.181
HSPA4	12.793	14.425	13.527	18.092	15.985	17.777	20.137	13.333	18.892	14.601	11.676	14.283	14.661	6.94
HSPA9	39.174	48.373	47.578	47.38	43.318	52.102	58.929	39.081	52.466	45.466	40.151	42.288	47.538	10.085
STIP1	19.699	24.364	20.88	35.008	33.32	27.94	27.395	20.658	33.277	22.758	18.658	21.743	21.758	13.47
HSP90AB1	251.347	334.997	237.183	361.248	317.441	370.466	424.425	253.033	380.755	274.064	192.744	230.737	271.362	81.307
DNAJA1	44.743	56.126	45.368	42.001	36.407	57.429	71.892	44.8	64.983	54.326	36.831	52.551	51.506	26.796
HSPH1	29.042	42.323	51.26	11.762	12.411	44.567	60.519	31.269	53.736	64.658	39.278	18.057	20.762	7.814
HSP90AA1	214.781	263.531	265.575	252.02	218.885	266.62	318.246	255.736	366.024	291.035	228.153	279.263	308.185	60.379
MCL1	24.537	20.394	23.479	23.917	24.348	20.084	19.599	20.325	28.365	21.5	20.561	34.239	32.044	203.897

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CHAPTER 4: DISCUSSION

The current hypothesis-generating study investigated the molecular mechanisms involved in PTSD on a whole-genome transcriptomic level by carrying out differentially expressed gene set analysis in a mixed ancestry South African population group. Gene expression data were generated through RNA-Seq, using whole blood samples isolated from participants with PTSD patients and trauma-exposed controls.

Through a series of analytical steps (Chapter 2: Section 2.8, 2.9, 2.10, 2.11) our investigation pursued the identification of several dysregulated co-expression networks in whole blood transcriptome of individuals diagnosed with PTSD. These networks consisted of genes with functions and phenotypes associated with anxiety and stress-related disorders such as PTSD, based on a knowledge driven approach using BORG. Biological evidences obtained for each gene which is directly or transitively linked to anxiety disorder and by extension PTSD included, but were not limited to; "behavioral fear response" (GO:0001662), "fear-induced aggressive behavior" (GO:0002122), "behavioral abnormality" (HP:0000708), "anxiety" (HP:0000739), "anxiety-related response" (MP:0001363), "abnormal cued conditioning behavior" (MP:0001454), "neurotrophic factor signaling pathway" (PW:0000571), "long-term depression" (PW:0000061) and "cortisol signaling pathway" (PW:0000569), all of which are key features of PTSD (see Table 2.2 for full list of GO, HPO, MPO and PO terms).

One such co-expression network consisted of four genes found to be significantly upregulated in anxiety and stress-related disorders (such as PTSD), all of which were involved in the biological process of "gene expression" (GO:0010467) (Table 3.3). These four upregulated genes were the ribosomal protein S6 gene (*RPS6*), the ribosomal protein L6 gene (*RPL6*), the ribosomal protein S3A gene (*RPS3A*) and the eukaryotic translation elongation factor 1 beta 2 gene (*EEF1B2*).

Additionally, we identified a large downregulated network comprising of 36 co-expressed genes that may be dysregulated in anxiety and stress-related disorders, including PTSD. We proceeded to focus on six highly inter-connected (genes co-expressing with several genes within the large co-expression network (Figure 3.5)), downregulated genes as dysregulation of these genes will most likely lead to a cascade of dysregulation in several genes connected within the network. These six genes included the eukaryotic translation initiation factor 4 gamma 1 gene (*EIF4G1*), the heat shock protein family A (Hsp70) member 4 gene (*HSPA4*), DEAH-box helicase 9 gene (*DHX9*), the B-cell lymphoma-2 associated transcription factor 1 gene (*BCLAF1*), thyroid hormone receptor associated protein 3 gene

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(*THRAP3*) and the myeloid cell leukemia 1 gene (*MCL1*). *In silico* tissue expression analysis confirmed that all ten of these genes, consisting of four upregulated co-expression network genes and six, highly connected downregulated co-expressed genes, were all expressed within the brain.

4.1 Upregulated gene set predicted to be involved in anxiety and stress-related disorders, including PTSD

RPS6

The ribosomal protein S6 (*RPS6*) gene was one of the four highly co-expressed, significantly upregulated genes found within our group of female PTSD patients. This ribosomal gene encodes for the small 40S subunit of the human 80S ribosome (Biever, Valjent & Puighermanal, 2015). Compared to whole blood tissue expression levels, high levels of *RPS6* expression are found within the spinal cord and substantia nigra. The highest expression levels, based on GTEx database, were found in the amygdala (Table 3.10), which plays a central role in fear response, threat detection and especially in fear conditioning (Davis, 1992). *RPS6* was also implicated in the "Hypoxia-inducible factor 1 (HIF-1) signaling pathway (hsa04066)" (Table 3.5) and "BDNF signaling pathway" (WP2380) (Table 3.6). This is of interest as HIF-1 functions as a master regulator of oxygen homeostasis and is involved in major pathological processes such as cardiovascular disease, inflammation and cancer, which have been associated with the formation of reactive oxygen species (ROS) (Niecknig *et al*., 2012). Interestingly Zieker *et al*., (2007) identified the downregulation of both immune-related and ROS genes (*TXR1, SOD1, IL-16, IL-18* and *EDG1*) in whole blood of PTSD individuals (Zieker *et al*., 2007). Also of note is that the *BDNF* gene has been investigated as a candidate gene implicated in PTSD (Table 1.2). Therefore, the dysregulation in *RPS6* expression interacts with multiple pathways previously implicated in PTSD.

In addition, a recent longitudinal PTSD risk and resilience study, conducted to create a predictive biomarker, indicated that *RPS6* was downregulated in peripheral blood cells amongst pre-deployed, male marines (who would later go on to develop PTSD) compared to trauma-exposed controls (Glatt *et al*., 2013). *RPS6* was one of the 23 genes used as a gene-based diagnostic predictor of pre-deployed individuals who would later develop PTSD. The panel attained 85% accuracy in classifying those individuals in the training sample who would or would not go on to develop PTSD whilst an independent test cohort, of five cases and five comparison subjects, yielded a 70% accuracy. The current study indicates that *RPS6* was significantly upregulated in females diagnosed with PTSD compared to trauma-exposed controls. *This observation suggests an overcompensation mechanism where the downregulation of RPS6 may lead to an increased susceptibility for PTSD after trauma-*

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exposure, and subsequent upregulation of RPS6 gene and the development of PTSD. RPS6 was not identified as a significantly DEG in a subsequent study comparing post-deployment male marines with PTSD relative to trauma-exposed controls (Tylee *et al*., 2015). This variability needs additional investigation as sample size were similar for both Tylee *et al*., (2015) and the present study. However, the present study only included females, which may account for the variability observed as it has been proposed that differences in rates of PTSD may be mediated, in part, by circulating estrogen levels (Maddox *et al*., 2017).

Additionally, post-translational modification of the *RPS6* gene through phosphorylation has also been widely investigated within the neurosciences as this gene forms part of the mammalian target of rapamycin complex (mTOR) signalling activation and serves as marker for neuronal activity (Meyuhas, 2008, 2015; Mahoney *et al*., 2009). The mTOR signalling pathway, consists of two complexes including the mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2) (reviewed by Laplante & Sabatini, 2009). Inhibition of mTORC1 is known to reduce protein synthesis, affecting translation by phosphorylating p70 S6 kinase polypeptide 1 (S6K1), which in turn, phosphorylates downstream targets such as RPS6 and eukaryotic translation initiation factor 4 beta (eIF4B) (Raught *et al*., 2004). Additionally, mTORC1-dependent translation has been implicated in altered memory strength of auditory fear conditioning in mice (Huynh, Santini & Klann, 2014). The latter study demonstrated that mTORC1-dependent translation in the reconsolidation (memory retrieval) of fear memory required both eIF4F formation and S6K1 activation (Huynh, Santini & Klann, 2014). This is of interest to the present study, as it has been well documented that memory processes are impaired within PTSD individuals (Elzinga & Bremner, 2002; Layton & Krikorian, 2002; American Psychiatric Association, 2013). Moreover, mTORC1 has been linked to drug-seeking behaviour and inhibitory avoidance (Jobim *et al*., 2012; Lin *et al*., 2014) which is of interest as high co-morbidity exists between PTSD and illicit drug use (McCauley *et al*., 2012). Therefore, it could be postulated that the inhibition of mTORC1 (which could in part lead to the impairment of memory processes in PTSD individuals) affects the translation of S6K1 which in turn lead to the upregulation of *RPS6* as seen in our PTSD subjects.

RPL6

Interestingly, the longitudinal PTSD risk and resilience study (Glatt *et al*., 2013) in which *RPS6* was found to be downregulated in peripheral blood cells indicated that ribosomal protein L6 gene (*RPL6*) was also downregulated among pre-deployed, male marines (who had later developed PTSD post-deployment) compared to male trauma-exposed controls without PTSD (Glatt *et al*., 2013). *RPL6* (encoding a protein component for the large 60S ribosomal subunit) was one of the 23 genes used in

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the gene-based diagnostic predictor panel of pre-deployed individuals who would later develop PTSD. The *RPL6* gene, as with *RPS6*, was not identified in a subsequent study comparing post-deployment male marines with PTSD relative to trauma-exposed controls (Tylee *et al*., 2015). This contrasts with our study where *RPL6* was found to be significantly upregulated among females with PTSD compared to female trauma-exposed controls. *This observation may suggest that the downregulation of RPL6 (as with RPS6) at baseline may lead to an increased susceptibility for PTSD leading to the subsequent upregulation of RPL6 after exposure to trauma and leading to the eventual development of PTSD. Therefore, it is evident that both RPS6 and RPL6 perform a similar function in gene regulation which may be dysregulated due to an overcompensation mechanism as mentioned above.*

A recent study investigated the influence of anxiety- and depression-like states (through use of a chronic social defeat stress (CSDS) model) in male mice on ribosomal gene expression in brain regions using RNA-Seq (Smagin *et al*., 2016). The study identified the upregulation of several differential expression ribosomal protein L genes, including the upregulation of *RPL6*, in the hypothalamus of mice following CSDS. Smagin *et al*., (2016) hypothesised that this upregulation of ribosomal genes in the hypothalamus was most likely due to a feedback mechanism in response to CSDS and not a result of ribosomal gene dysfunction leading to development of anxiety- and depression-like states in mice (Smagin *et al*., 2016).

RPS3A

The *RPS3A* gene encodes for the ribosomal protein S3A, a component of the 40S ribosomal subunit involved in gene expression (GO:0010467) (Table 3.3). This ribosomal coding protein has not yet been directly implicated in anxiety- and stress related disorders, however the RSP3A gene has been found to be significantly upregulated in the peripheral blood of heavy drinkers one hour after experiencing psychological stress (Beech *et al*., 2014). Additionally, the *RPS3A* gene interacts with the Heat Shock Protein 90 Alpha Family Class A Member 1 (*HSP90AA1*) gene, a member of the cortisol signalling pathway (Stelzl *et al*., 2005), a pathway which has been linked to PTSD (Chapter 1: Section 1.2.1). Notably, the gene expression levels of *HSP90AA1* were found to be significantly downregulated in our set of DEGs between our PTSD patients compared to trauma-exposed controls. Additionally, *RPS3A* is co-expressed with *RPS6* and *RPL6* (Figure 3.5), all ribosomal encoding genes which play an integral role in the translational mechanism. Therefore, the dysregulation of these ribosomal encoding genes may explain, to some extent, the differences seen in gene expression between PTSD patients and trauma-exposed controls. However, additional research is required to clarify the exact mechanism involved in the differential expression of *RPS3A* in PTSD individuals.

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EEF1B2

The eukaryotic translation elongation factor 1 beta 2 gene encodes for a translation elongation factor involved in the transfer of aminoacylated tRNAs to the ribosome (von der Kammer *et al*., 1991). According to the ontological results of the BORG analysis, *EEF1B2* direct, biochemical interaction with Dopamine Receptor D3 (*DRD3*) gene (Cho *et al*., 2003), a member of the dopamine signalling pathway that has been mostly investigated within schizophrenia patients (Nunokawa *et al*., 2010). Mouse knockout models of *DRD3* have also been associated with a decreased anxiety-related response phenotype as well as an increase in thigmotaxis which is the motion or orientation of an organism in response to a touch stimulus (Halberstadt & Geyer, 2009). This increase in thigmotaxis is of note as one of the core symptoms of PTSD is an exaggerated startle response (Grillon *et al*., 1996).

EEF1B2 also interacts with Unc-51 Like Autophagy Activating Kinase 2 (*ULK2*), which, in gene knockout mouse models, leads to an increase in anxiety-related response (http://www.informatics.jax.org /reference/J:103485). We did not find literature linking the *EEF1B2* gene directly to PTSD; however, the *EEF1B2* gene is involved in the transcriptional regulation of several genes, including genes *DRD3,* associated with an anxiety-related response, and *ULK2* associated with increased in thigmotaxis. Thus, dysregulation of *EEF1B2* may result in inefficient transcriptional regulation of these aforementioned genes, which are associated with responses (such as exaggerated startle response) often seen in PTSD patients.

Summary of upregulated genes predicted to be involved in anxiety and stress-related disorders, including PTSD

In whole blood, major components of ribosomes; the small ribosomal subunit that reads the RNA (Rps) and the large subunit that connects amino acids to form a polypeptide chain (Rpl), showed differential expression in our group of female PTSD patients. Finding a direct association between the upregulation of these ribosomal genes could facilitate our understanding of the cause and the consequence of the processes involved in PTSD. However, it is probable that a cascade of systemic changes at a whole blood level will lead to changes in the expression of genes involved in the development PTSD.

Co-expression analysis showed a strong co-expression of *RPS6, RPL6, RPS3A* and *EEF1B2* genes, which were highly upregulated in the present study. These genes are all involved in the translational process and thus involved within protein expression. *We, therefore, hypothesise that these upregulated genes most likely represent a very specific fine tuning of the translational machinery in the response to trauma. This either predisposes to PTSD [refer to RPS6, RPL6 (Glatt et al., 2013),*

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EEF1B2], or is a response to PTSD [refer to RPL6 (Smagin et al., 2016)] once manifested. Furthermore, this may represent a predictive biosignature that can be tested in a further study [refer to RPS6, RPL6 (Glatt et al., 2013)].

4.2 Downregulated genes predicted to be involved in anxiety and stress-related disorders, including PTSD

EIF4G1

The Eukaryotic translation initiation factor 4 gamma 1 (*EIF4G1*) gene encodes for a component (eIF4G) of the eIF4F protein complex, which consists of three subunits, namely eIF4G, eIF4E (bind with 5'cap of mRNA) and eIF4A (Pelletier *et al*., 2015). The eIF4F protein complex functions as a rate-limiting step in the initiation phase of protein synthesis, facilitating the recruitment of mRNA to the ribosome (reviewed by Hinnebusch & Lorsch, 2012) and is thus a member of the "translation initiation pathway" (PW:0000580).

Interestingly, *EIF4G1* has also been associated with the human phenotype of "sleep disturbance" (HP:0002360), providing a possible link to PTSD as this phenotype is a common symptom displayed within PTSD patients (American Psychiatric Association, 2013). Additionally, a recent study by Huynh *et al*., (2014), investigated memory strength of auditory fear conditioning in mice which demonstrated that eIF4F formation, along with S6K1 activation (see Section 4.1 RPS6) were required for fear memory reconsolidation in mTORC1-dependent translation (Huynh, Santini & Klann, 2014). Other studies also demonstrated that the inhibition of mTORC1 blocks the consolidation (process of converting information from short-term memory into long-term memory) and reconsolidation of cued fear memory, but inhibition of eIF4E–eIF4G interactions blocks only consolidation (Hoeffer *et al*., 2011; Mac Callum *et al*., 2014). In the present study, the *EIF4G1* gene, which encodes a component of the eIF4G protein, was found to be significantly downregulated between our PTSD patients and trauma-exposed controls. *It is, therefore, hypothesised that dysregulation of the EIF4G1 gene might lead to the inhibition of the eIF4E–eIF4G interactions, possibly blocking the process of memory consolidation in individuals with PTSD.*

HSPA4

The heat shock protein family A (Hsp70) member 4 (HSPA4) gene encodes the heat shock 70 kDa protein 4, which is implicated in the "cortisol signalling pathway" (PW:0000569). HSPA4 was also identified by our BORG query and KEGG analysis to be a member of antigen processing and presentation pathway (Table 3.9). Our anxiety BORG analysis indicated interactions between HSPA4

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and TNF receptor associated factor 6 (TRAF6), a member of the neurotrophic factor signalling pathway. Knockout studies of *TRAF6* associated the gene in immune system regulations such as the positive regulation of interleukin-2 and T cell cytokine production within mouse and rat models (Sun *et al*., 2004). This is of note as T cells are postulated to have a neuroprotective function during conditions of injury, infection and in emotional or psychological stress (Miller, Maletic & Raison, 2009; Schwartz & Shechter, 2010).

The *HSPA4* gene furthermore interacts with RAF1 (Raf-1 Proto-Oncogene, Serine/Threonine Kinase) gene, which forms part of the neurotrophic factor signalling pathway and has a function in the long-term depression pathway (Yamamoto *et al*., 2012). Both these pathway ontology terms were found to be overrepresented within our downregulated biologically relevant gene-set. Moreover, HSPA4 interacts with both Inhibitor of Kappa Light Polypeptide Gene Enhancer in B-Cells, Kinase Gamma (IKBKG) and CD40 which are involved in the positive regulation of type I interferon and interleukin-12 production respectively (McKee & Pearce, 2004). Other interactions include the PPP1CA gene and ESR1 gene, which are both members of the serotonin signalling pathway via receptors engaging in G alphas protein family.

Recently a study was conducted to investigate epigenetic mechanism of pregnant PTSD rats and the subsequent development of their offspring (Zhang *et al*., 2016). The study utilised a single prolonged stress (SPS) model to simulate PTSD in pregnant rats thereafter both the dysregulation of methylation and gene expression in the offspring of stress-induced rats were investigated. Interestingly, the gene expression analysis indicating that *HSPA4* was one of the significantly downregulated genes in the offspring of stress-induced rats 30 days after birth (Zhang *et al*., 2016). Our study found similar results to that of Zhang *et al*., (2016), indicating that *HSPA4* was significantly downregulated in PTSD patients compared to trauma-exposed controls. *Therefore, we hypothesise that gene expression changes, such as the downregulation of HSPA4, could possibly be due to the stress-induced dysregulation of whole-genome methylation. The dysregulation at a genomic level subsequently contributed to the dysregulation of genes such as HSPA4*.

DHX9

The DEAH-box helicase 9 (*DHX9*) is a transcription activator that encodes for a member of the DEAH-containing family of RNA helicases. The enzyme encoded by this gene is implicated in ATP-dependant unwinding of double stranded RNA complexes and therefore functions as a transcriptional regulator (reviewed by Jankowsky, 2011). The *DHX9* gene has a function in “ATP binding” (GO:0005524) (Table 3.4) and is also involved in “circadian rhythm” (GO:0007623). This is of interest as sleep disturbances are common among patients with PTSD (Pace-Schott *et al*., 2015).

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Moreover, sleep-related symptoms of PTSD are included in the DSM-5 (American Psychiatric Association, 2013). *Therefore, gene expression changes of DHX9 may alter circadian rhythms leading to sleep disturbances associated with PTSD.*

The *DHX9* gene is also a member of the "aldosterone signalling pathway" (PW:0000568) (Yang & Fuller, 2011) which is of interest as anxiety increases aldosterone levels (Hlavacova & Jezova, 2008). Gene ontology of abnormal T cell differentiation as well as abnormal embryogenesis/ development in mice has also been reported (Zhu *et al.*, 2012). This is significant as T cells are hypothesised to have a neuroprotective function during conditions such as infection, injury or psychological stress (Miller, Maletic & Raison, 2009; Schwartz & Shechter, 2010). *Therefore, the differential expression of DHX9 found in individuals with PTSD, in the present study, may, in part, lead to abnormal T cell differentiation which dysregulates neuroprotective functioning under extreme psychological stress (such as that which occurs in the experience of traumatic events).*

The *DHX9* gene is furthermore involved in positive regulation of type I interferon production (Li *et al.*, 2011). *DHX9* also interacts with RELA Proto-Oncogene, NFkB Subunit (RELA), involved in neurotrophic factor signalling pathway and involved in the positive regulation of type I interferon production (Liu *et al.*, 2003). The gene furthermore interacts with Jun Proto-Oncogene, AP-1 Transcription Factor Subunit (JUN) implicated in the neurotrophic factor signalling pathway which is also a role player in learning (Tischmeyer *et al.*, 1994) and BRCA1 involved in the positive regulation of vascular endothelial growth factor production (Singh *et al.*, 2013). The downregulation of this transcription activator is in line with previous gene expression studies reporting a reduction in expression of transcription activators in peripheral blood mononuclear cells of PTSD patients (Segman *et al.*, 2005; Neylan *et al.,* 2011).

BCLAF1

The B-cell lymphoma-2 associated transcription factor 1 (*BCLAF1*) gene encodes a transcriptional repressor that interacts with proteins of the B-cell lymphoma 2 (Bcl-2) family, which play a prominent role in apoptosis and the enhancement of cell survival in a variety of cells including lymphohematopoietic and neural cells (Kasof, Goyal & White, 1999). The overexpression of *BCLAF1* induces apoptosis, which in turn can be suppressed by co-expression of BCL2 proteins (Kasof, Goyal & White, 1999). Recent studies have implicated *BCLAF1* in processes of RNA metabolism (Bracken *et al.*, 2008; Sarras, Alizadeh Azami & McPherson, 2010) and T cell activation (McPherson *et al.*, 2009; Kong *et al.*, 2011). The *BCLAF1* gene is also implicated in carcinoma and knockout causes "decreased CD8-positive, alpha-beta T cell number" (MP:0008079) as well as "decreased T cell proliferation" (MP:0005095). As mentioned previously (see Section 4.2 *DHX9*) T

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cells are hypothesized to have a neuroprotective function during conditions such as infection, injury or psychological stress (Miller, Maletic & Raison, 2009; Schwartz & Shechter, 2010). *Thus, differential expression of BCLAF1 found in the present study may to some extent lead to abnormal T cell functioning during extreme psychological stress, leading to the development of PTSD. BCLAF1* is furthermore involved in the "regulation of DNA-templated transcription in response to stress" (GO:0043620) and has functions in DNA binding, poly(A) RNA binding and protein binding.

As mentioned previously, the *BCLAF1* gene interacts with the Bcl-2 family, including the *Bcl-2* gene which is involved in gene ontology of "behavioural fear response" within animal models (Einat, Yuan & Manji, 2005). The study by Einat *et al*., (2005) was designed to explore behavioural models of psychiatric disorders in male mice through the targeted mutation of the *Bcl-2* gene (heterozygote mice). The results demonstrated an increase in anxiety-like behaviours in mice with reduced mitochondrial Bcl-2 levels compared to that of wild type mice. These results possibly indicate the critical role of *Bcl-2* in the aetiology of anxiety disorders. Additionally, *BCLAF1* interacts with tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (*YWHAG*), a member of a neurotrophic factor signalling pathway (Jin *et al*., 2004) which was also a gene significantly downregulated within the present study and found to be biologically-relevant based on the anxiety BORG database.

THRAP3

The thyroid hormone receptor associated protein 3 (*THRAP3*) gene encodes the protein thyroid hormone receptor associated protein 3, also known as TRAP150 (Ito *et al*., 1999). The protein is a subunit of the TRAP/Mediator complex, which is implicated in transcriptional regulation (Beli *et al*., 2012) and an important role player in the pre-processing of mRNA as it forms part of a component of the spliceosome (Enrichment analysis identified several genes implicated in the spliceosome based on the KEGG pathway Table 3.5). Moreover, the protein encoded for by THRAP3 shares sequencing similarities with that of the BCLAF1 transcriptional repressor protein encoded for by the *BCLAF1* gene. Interestingly, both *THRAP3* and *BCLAF1* interact with the human gene *YWHAG,* which is a member of the neurotrophic factor signalling pathway found to be significantly downregulated within the present study and found to be biologically-relevant based on the anxiety BORG database. The THRAP3 protein acts as a co-activator which promotes transcriptional activation and binding to circadian target genes which positively regulates circadian rhythm (Lande-Diner *et al*., 2013). These circadian clock proteins play an integral role in sleep. *It is hypothesised that the dysregulation of a co-activator protein, such as THRAP3, may lead to an impaired functioning of circadian rhythms*

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(Lande-Diner *et al*., 2013). *This in turn could induce sleep disturbances such as insomnia, a common symptom in patients with PTSD* (American Psychiatric Association, 2013).

The *THRAP3* gene also has a function in thyroid hormone receptor binding (Ito *et al*., 1991), bringing into question the hypothalamic-pituitary-thyroid (HPT) axis involvement in PTSD. The HPT axis is involved in homeostasis by regulating thyroid hormone levels within the blood. Trauma is known to trigger thyroid abnormalities; however, research on the relationship between the HPT axis and PTSD remains limited (Sherin & Nemeroff, 2011). Additionally, stress/cortisol influences the feedback loop of thyroid hormones (Walter *et al*., 2012) however, this process involves complex endocrinology which requires further investigation in future PTSD studies.

MCL1

Although the myeloid cell leukemia 1 (*MCL1*) gene was not found to be significantly differentially expressed between PTSD patients and control groups, it is included in the discussion as it was found to be a highly connected gene within the large downregulated co-expressed network using COXPRESdb (Figure 3.5). The *MCL1* gene encodes for an anti-apoptotic protein and is a member of Bcl-2 family (Czabotar *et al*., 2011). As mentioned previously (see Section 4.2 *BCLAF1*) the Bcl-2 family, plays a prominent role in supressing apoptosis and the enhancement of cell survival (Kasof, Goyal & White, 1999) in a variety of cells including lymphohematopoietic and neural cells (Maurer *et al*., 2006).

Recently a PTSD study, employing a single-prolonged stress (SPS) method in rats, investigated apoptosis-related gene expression of Bcl-2 and Bcl2-associated X (Bax) in the mPFC. The results indicated that SPS stimulation increased the number of apoptotic neurons, up-regulated the expressions of Bcl-2 and Bax, and altered the Bcl-2/Bax ratio in the mPFC of PTSD rats (Li, Han & Shi, 2013). However, additional investigation is needed, including the dysregulation of *MCL1* in PTSD patients, as the role of apoptosis in the pathogenesis of PTSD is not yet certain (Li, Han & Shi, 2013).

Summary of downregulated genes predicted to be involved in anxiety and stress-related disorders, including PTSD

The six highly connected downregulated genes form part of a large co-expressed network. This is interesting in itself, as downregulation of the expression of such a large network is likely to have biological and hence biomedical effects. Several of the highly connected genes discussed, are implicated in transcriptional regulation (*DHX9, BCLAF1* and *THRAP3*), which correlates to the findings of previous studies (Segman *et al*., 2005; Neylan *et al*., 2011), where an overall reduction of

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transcriptional activators (which in turn regulates gene expression) was observed in the peripheral blood of psychologically distressed victims (*PF4, HIST1H2AC* and *SDPR*) (Neylan *et al*., 2011). This general reduction of transcription activators in response to stress may, in part, explain the differences in gene expression signatures observed between our PTSD and trauma-exposed control subjects (Segman *et al*., 2005).

Immune dysregulation has been found to result in vulnerability to PTSD via alterations in brain function (Lanius *et al*., 2010). The brain is supported by the immune system with immune functioning playing an essential role in learning and memory under basal conditions, and supports optimal stress-coping responses (Molina-Holgado & Molina-Holgado, in press; Su, Zhang & Schluesener, 2010; Yirmiya & Goshen, 2011). Acute stress, in both humans and animals, results in enhanced cell-mediated immunity (Dhabhar & Mcewen, 1997), increased levels of proinflammatory cytokines and increased blood-brain barrier permeability (Škultétyová, Tokarev & Ježová, 1998). This could result in increased neuroinflammation, which, in turn, has been associated with disorders such as PTSD (reviewed by Zass *et al.,* 2017). Our results, are in line with previous findings of dysregulated expression profiles of immune-related genes in stress-related disorders, such as PTSD (Zieker *et al*., 2007; Yehuda *et al*., 2009; Neylan *et al*., 2011; Glatt *et al*., 2013; Breen *et al*., 2015). This is of note, as two of the highly connected downregulated genes identified in this study (*DHX9* and *BCLAF1*) have an immune-related function.

Additionally, several of the downregulated genes (*EIF4G1, DHX9* and *THRAP3*) within the present study were previously found to be associated with sleep disturbances (Pace-Schott *et al*., 2015) and circadian rhythms (Lande-Diner *et al*., 2013). As mentioned previously, this is of interest to the present study, as sleep disturbances are common amongst PTSD patients (American Psychiatric Association, 2013). However, it is of note that both circadian rhythms and PTSD consist of complex gene networks and biological interactions (Landgraf, McCarthy & Welsh, 2014). Therefore, downregulation of *EIF4G1, DHX9* and *THRAP3* possibly explains only a minor part of the broad networks overlapping in PTSD and circadian rhythms.

4.3 Overall summary of up- and downregulated gene sets

In summary, differential gene expression analyses in the current study revealed the upregulation of genes involved in translational process of protein expression and the downregulation of genes involved in immune-related function, circadian rhythm and transcriptional regulation. These preliminary findings provide novel insight into the underlying genetic mechanism of PTSD in South African population. The upregulation of translational machinery identified within the whole blood of

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PTSD patients (compared to trauma-exposed controls) occurs after exposure to trauma, and may lead to the eventual development of PTSD, or is the consequence of PTSD once it has already manifested.

Additionally, the general reduction in whole blood expression of transcriptional regulators may explain the differences in gene expression signatures observed between our PTSD patients and trauma-exposed control subjects. However, this reduction could possibly be due to the stress-induced dysregulation of whole-genome methylation, resulting in the dysregulation of gene transcripts related to transcriptional activation, intracellular signalling pathways and apoptosis. The current study, provides evidence supporting a blood transcriptomic response worth investigating in PTSD. It does however remain unclear whether the changes observed in the whole blood transcriptome are merely informative of the development of PTSD or whether these changes are also relevant to the pathogenesis of PTSD.

4.4 Limitations of study

The present study enabled the investigation of whole blood gene expression levels between PTSD patients and trauma-exposed controls, through use of the powerful next-generation sequencing technology, RNA-Seq. Our study encountered several limitations, including a cross-sectional design and a limited sample size consisting of 48 study participants. The sample size was in line with several other microarray gene expression studies including that of Glatt *et al*., (2013) (N=25 eventual PTSD cases and N=25 trauma-exposed controls) and Tylee *et al*., (2015) (N=25 PTSD patients vs. N=25 trauma-exposed controls). However, a recent RNA-Seq study used a larger sample size (N=47 PTSD patients vs. N=47 trauma-exposed controls) in order to investigate differential expression in patients with PTSD (Breen *et al*., 2015). Nevertheless, these abovementioned studies did not take additional known risk factors for PTSD, such as family history, childhood exposure to trauma and pre-existing mental disorders into account. Although the present study excluded serious mental disorders, such as schizophrenia and bipolar disorders, major depressive disorder (MDD) given its high comorbidity with PTSD was not excluded provided that MDD was not the primary diagnosis. Five participants with PTSD in this study had comorbid MDD.

Another limitation of the study is the use of whole blood samples in a disease which is thought to be primarily a brain disorder. However, investigating gene expression levels within the brains of living human participants with PTSD is not currently possible. Post-mortem brains of PTSD patients could be investigated, however this will not be without its own challenges, including differential gene expression after death. Additionally, several studies have identified that peripheral blood gene expression signatures could be valuable in identifying mental disorder such as PTSD (Segman *et al*.,

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2005; Zieker *et al*., 2007; Yehuda *et al*., 2009; Neylan *et al*., 2011; Glatt *et al*., 2013; Breen *et al*., 2015; Tylee *et al*., 2015). For these reasons, investigating blood biomarkers for brain disorders remains a challenging, yet practical and less invasive, approach.

A further limitation is that the RNA-Seq procedure generated short reads of 50bp, which was later shortened to 42bp after the removal of the RNA-Seq indexes. This limited the potential mapping accuracy of reads to the reference genome. However, it has been reported that at 50bp, only a small percentage (<0.01%) of reads will map to more than one location of the reference genome (Korpelainen *et al*., 2014). Therefore, a 50bp read length should have been adequate for our preliminary differential expression study. Additionally, the present study utilised a sequencing depth of approximately 50 million paired end reads, enabling a greater accuracy in the quantification of up- and down- regulated genes.

It is further important to note that this preliminary study only reported on data at a transcriptomic level, roughly explaining potential proteins expression associated with PTSD development. However, our results should be followed up on a proteomic level before conclusions based on protein levels can be made. Additionally, epigenetic mechanisms and other non-coding RNAs could have contributed to differential gene expression however, this was beyond the scope of the present study.

The validation of a subset of DEG through qPCR was not done within the present preliminary sub-study as the sample size was relatively small. Results will be validated by use of qPCR if subsequent SHARED ROOTS transcriptomic sub-studies identify the same genes as in this preliminary study, as this would provide justification for the validation.

4.5 Future studies

Future work should include miRNA profiling, which may identify a more robust signature as miRNAs are evolutionarily conserved and involved in various intricate processes including the stress response (He *et al*., 2007). Therefore, any miRNAs causing the downregulation of mRNAs will be upregulated themselves and would thus present potentially measurable blood-based biomarkers for PTSD. Additionally, RNA-Seq can be used to investigate the shared genetic factors between PTSD and other psychiatric disorders enabling the development of better diagnostic and treatment methods for PTSD patients.

We also recommended that future RNA-Seq studies (especially transcriptomic studies of the SHARED ROOTS project) utilise a longer read length (75bp-100bp), as this is essential for identifying possible splice variants which could provide additional insight into differential gene

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expression (Wang *et al*., 2009). Additionally, the differential expression of the transcriptional and translational machinery, identified within the present study, are most likely due to variations on a genomic level. Therefore, a need exists for the incorporation of larger sample sizes and multiple approaches, combining genomic, transcriptomic, epigenetic and neuroimaging data as is being done in the SHARED ROOTS project. These approaches will enable specific variant detection as well as allele-specific expression detection. Future transcriptomic work of the SHARED ROOTS project will focus on the differential gene expression of PTSD patients and trauma-exposed controls with metabolic syndrome in order to test for genes linked within this co-morbidity.

4.6 Conclusion

The current study was conducted as part of a larger interdisciplinary South African Medical Research Council (MRC) flagship project SHARED ROOTS. This preliminary investigation allowed for the identification of a set of DEGs between PTSD patients and trauma-exposed controls using the whole genome transcriptomic approach, RNA-Seq.

PTSD remains a complex, neuropsychiatric disorder underpinned by multi-factorial interactions. However, based on multiple sources of evidence, the present study highlights potential biological and biomedical roles of four co-expressed upregulated genes (*RPL6, RPS6, RPS3A* and *EEF1B2*) and six highly connected co-expressed downregulated genes (*DHX9, BCLAF1, THRAP3, EIF4G1, HSPA4* and *MCL1*), which were identified as potentially relevant gene candidates contributing to the pathology of PTSD. Additionally, the data provides supporting evidence of a blood transcriptomic response worth investigating in a future study.

These preliminary findings provide novel insight in underlying genetic expression of PTSD in South African population. However, the candidate genes identified within the present study do warrant further research to test the hypotheses generated.

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APPENDIX I

Index labels used in pooled RNA sequencing data generated

Table I.1: Pooled RNA sequencing data identifying sequences of index labels. Tables indicates the pool number and unique index labels (with sequences) used for each of the sample.

Sample Name	Index 1	Index 2	Pool	Seq Index 1	Seq Index 2
SR148	701	502	1	ATTACTCG	ATAGAGGC
SR066	702	502	1	TCCGGAGA	ATAGAGGC
SR150	703	502	1	CGCTCATT	ATAGAGGC
SR146	704	502	1	GAGATTCC	ATAGAGGC
SR072	705	502	1	ATTCAGAA	ATAGAGGC
SR001	706	502	1	GAATTCGT	ATAGAGGC
SR186	707	502	1	CTGAAGCT	ATAGAGGC
SR230	708	502	1	TAATGCGC	ATAGAGGC
SR176	711	502	1	TCTCGCGC	ATAGAGGC
SR052	712	502	1	AGCGATAG	ATAGAGGC
SR077	701	503	2	ATTACTCG	CCTATCCT
SR135	702	503	2	TCCGGAGA	CCTATCCT
SR164	703	503	2	CGCTCATT	CCTATCCT
SR113	704	503	2	GAGATTCC	CCTATCCT
SR156	705	503	2	ATTCAGAA	CCTATCCT
SR187	706	503	2	GAATTCGT	CCTATCCT
SR105	707	503	2	CTGAAGCT	CCTATCCT
SR158	708	503	2	TAATGCGC	CCTATCCT
SR016	709	503	2	CGGCTATG	CCTATCCT
SR058	710	503	2	TCCGCGAA	CCTATCCT
SR190	711	503	3	TCTCGCGC	CCTATCCT
SR170	712	503	3	AGCGATAG	CCTATCCT
SR166	701	504	3	ATTACTCG	GGCTCTGA
SR065	702	504	3	TCCGGAGA	GGCTCTGA
SR015	703	504	3	CGCTCATT	GGCTCTGA
SR019	704	504	3	GAGATTCC	GGCTCTGA
SR096	705	504	3	ATTCAGAA	GGCTCTGA
SR132	706	504	3	GAATTCGT	GGCTCTGA
SR038	707	504	3	CTGAAGCT	GGCTCTGA
SR048	708	504	3	TAATGCGC	GGCTCTGA
SR214	709	504	4	CGGCTATG	GGCTCTGA
SR193	710	504	4	TCCGCGAA	GGCTCTGA
SR140	711	504	4	TCTCGCGC	GGCTCTGA
SR209	712	504	4	AGCGATAG	GGCTCTGA
SR075	701	505	4	ATTACTCG	AGGCGAAG
SR139	702	505	4	TCCGGAGA	AGGCGAAG

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SR279	703	505	4	CGCTCATT	AGGCGAAG
SR177	704	505	4	GAGATTCC	AGGCGAAG
SR013	705	505	4	ATTCAGAA	AGGCGAAG
SR098	706	505	4	GAATTCGT	AGGCGAAG
SR055	709	505	5	CGGCTATG	AGGCGAAG
SR006	711	505	5	TCTCGCGC	AGGCGAAG
SR089	712	505	5	AGCGATAG	AGGCGAAG
SR082	701	506	5	ATTACTCG	TAATCTTA
SR109	702	506	5	TCCGGAGA	TAATCTTA
SR119	703	506	5	CGCTCATT	TAATCTTA
SR080	706	506	6	GAATTCGT	TAATCTTA
SR092	709	506	6	CGGCTATG	TAATCTTA

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APPENDIX II

Biologically relevant differentially expressed gene as identified by anxiety BORG analyses

The current study of focused on the DEGs between PTSD vs. trauma-exposed control groups. Table I.1 depicts the DEG identified through DESEQ2 analysis which were found to be biologically relevant according to our anxiety BORG analyses. The negative fold changes imply that gene expression levels in the PTSD group is lower than that in the control group; positive fold changes imply that gene expression levels in the PTSD group is higher than that in the control group.

Table II.1: Biologically significant differentially expressed genes between PTSD patients and trauma-exposed controls (as identified by anxiety BORG analyses). The negative fold changes indicate that genes were downregulated whilst a positive fold change indicates upregulated genes in the PTSD group compared to the control group.

Gene	Name	log2 Fold-Change	p-adjusted
CX3CR1	C-X3-C motif chemokine receptor 1	-0.517	0.008
ANXA5	annexin A5	-0.477	0.007
DPYSL2	dihydropyrimidinase like 2	-0.464	0.008
GBP1	guanylate binding protein 1	-0.438	0.020
ANXA3	annexin A3	-0.436	0.032
NCL	nucleolin	-0.423	0.008
PRF1	perforin 1	-0.411	0.033
IQGAP1	IQ motif containing GTPase activating protein 1	-0.410	0.023
HSPA9	heat shock protein family A (Hsp70) member 9	-0.409	0.007
IFI16	interferon gamma inducible protein 16	-0.409	0.015
ACSL4	acyl-CoA synthetase long-chain family member 4	-0.409	0.010
PDIA3	protein disulfide isomerase family A member 3	-0.408	0.007
OAT	ornithine aminotransferase	-0.407	0.022
PARP14	poly(ADP-ribose) polymerase family member 14	-0.406	0.025
LCP1	lymphocyte cytosolic protein 1	-0.404	0.015
PIK3AP1	phosphoinositide-3-kinase adaptor protein 1	-0.399	0.014
NOLC1	nucleolar and coiled-body phosphoprotein 1	-0.399	0.008
AHR	aryl hydrocarbon receptor	-0.393	0.014
PLSCR1	phospholipid scramblase 1	-0.393	0.041
EZR	ezrin	-0.392	0.014
HSP90B1	heat shock protein 90 beta family member 1	-0.390	0.008
TXNIP	thioredoxin interacting protein	-0.386	0.017
F2R	coagulation factor II thrombin receptor	-0.386	0.026
MSN	moesin	-0.385	0.018
ITGB1	integrin subunit beta 1	-0.374	0.019
CR1	complement component 3b/4b receptor 1 (Knops blood group)	-0.373	0.039
HSP90AB1	heat shock protein 90 alpha family class B member 1	-0.371	0.011

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TLN1	talin 1	-0.369	0.045
DDX21	DEAD-box helicase 21	-0.368	0.010
F5	coagulation factor V	-0.367	0.048
VCL	vinculin	-0.367	0.031
IL18RAP	interleukin 18 receptor accessory protein	-0.364	0.045
SYT11	synaptotagmin 11	-0.361	0.023
CTR9	CTR9 homolog, Paf1/RNA polymerase II complex component	-0.359	0.010
HSPA4	heat shock protein family A (Hsp70) member 4	-0.357	0.008
HSP90AA1	heat shock protein 90 alpha family class A member 1	-0.356	0.015
MEF2C	myocyte enhancer factor 2C	-0.355	0.018
MEF2A	myocyte enhancer factor 2A	-0.355	0.009
SIGLEC10	sialic acid binding Ig like lectin 10	-0.354	0.038
MARCKS	myristoylated alanine rich protein kinase C substrate	-0.353	0.048
HSPA5	heat shock protein family A (Hsp70) member 5	-0.351	0.022
TPR	translocated promoter region, nuclear basket protein	-0.351	0.015
CYBB	cytochrome b-245 beta chain	-0.350	0.037
HIF1A	hypoxia inducible factor 1 alpha subunit	-0.349	0.023
KLRD1	killer cell lectin like receptor D1	-0.345	0.046
CAST	calpastatin	-0.345	0.008
TLR5	toll like receptor 5	-0.342	0.046
MFAP1	microfibrillar associated protein 1	-0.341	0.010
RANBP2	RAN binding protein 2	-0.340	0.010
LRRK2	leucine rich repeat kinase 2	-0.339	0.045
TROVE2	TROVE domain family member 2	-0.339	0.010
EIF3A	eukaryotic translation initiation factor 3 subunit A	-0.338	0.014
GBP2	guanylate binding protein 2	-0.337	0.022
SLK	STE20 like kinase	-0.336	0.019
WBP11	WW domain binding protein 11	-0.336	0.008
ANXA2	annexin A2	-0.336	0.032
POLR2B	RNA polymerase II subunit B	-0.335	0.008
ANXA6	annexin A6	-0.334	0.016
HTATSF1	HIV-1 Tat specific factor 1	-0.333	0.011
NBN	nibrin	-0.332	0.023
DDB1	damage specific DNA binding protein 1	-0.331	0.039
XRCC6	X-ray repair cross complementing 6	-0.331	0.017
HNRNPK	heterogeneous nuclear ribonucleoprotein K	-0.329	0.016
PRKAR1A	protein kinase cAMP-dependent type I regulatory subunit alpha	-0.328	0.026
PARP1	poly(ADP-ribose) polymerase 1	-0.327	0.020
COPB2	coatomer protein complex subunit beta 2	-0.326	0.015
ENO1	enolase 1	-0.322	0.023
SETD7	SET domain containing lysine methyltransferase 7	-0.321	0.010
CHD1	chromodomain helicase DNA binding protein 1	-0.320	0.015
PPP1R12A	protein phosphatase 1 regulatory subunit 12A	-0.320	0.016
TCF4	transcription factor 4	-0.319	0.028
CCR2	C-C motif chemokine receptor 2	-0.317	0.046
JAK1	Janus kinase 1	-0.316	0.016
EIF4G1	eukaryotic translation initiation factor 4 gamma 1	-0.316	0.036
STAG2	stromal antigen 2	-0.312	0.024
KPNB1	karyopherin subunit beta 1	-0.310	0.024

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YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein beta	-0.310	0.012
HSPH1	heat shock protein family H (Hsp110) member 1	-0.309	0.014
SF3B2	splicing factor 3b subunit 2	-0.309	0.021
TES	testin LIM domain protein	-0.307	0.008
ICMT	isoprenylcysteine carboxyl methyltransferase	-0.306	0.016
TM9SF2	transmembrane 9 superfamily member 2	-0.306	0.034
VAV3	vav guanine nucleotide exchange factor 3	-0.306	0.011
THRAP3	thyroid hormone receptor associated protein 3	-0.306	0.028
GTF2A1	general transcription factor IIA subunit 1	-0.304	0.010
CANX	calnexin	-0.304	0.028
FYB	FYN binding protein	-0.301	0.016
REST	RE1 silencing transcription factor	-0.301	0.011
TOP2B	topoisomerase (DNA) II beta	-0.300	0.014
VPS35	VPS35 retromer complex component	-0.298	0.020
CHD4	chromodomain helicase DNA binding protein 4	-0.298	0.032
PGK1	phosphoglycerate kinase 1	-0.298	0.024
GNB4	G protein subunit beta 4	-0.297	0.032
COPB1	coatomer protein complex subunit beta 1	-0.295	0.011
STAT3	signal transducer and activator of transcription 3	-0.295	0.043
RFC1	replication factor C subunit 1	-0.294	0.015
ATP6AP2	ATPase H+ transporting accessory protein 2	-0.292	0.043
IRF4	interferon regulatory factor 4	-0.292	0.033
LDHA	lactate dehydrogenase A	-0.292	0.019
XRCC5	X-ray repair cross complementing 5	-0.291	0.015
TSPYL1	TSPY like 1	-0.291	0.015
HIPK1	homeodomain interacting protein kinase 1	-0.291	0.038
SP4	Sp4 transcription factor	-0.291	0.014
PLEK	pleckstrin	-0.291	0.024
CPNE3	copine 3	-0.290	0.031
ATP2B4	ATPase plasma membrane Ca2+ transporting 4	-0.289	0.046
CLTC	clathrin heavy chain	-0.289	0.032
NCOA3	nuclear receptor coactivator 3	-0.288	0.012
DNAJA1	DnaJ heat shock protein family (Hsp40) member A1	-0.288	0.026
XRN2	5'-3' exoribonuclease 2	-0.288	0.021
EPRS	glutamyl-prolyl-tRNA synthetase	-0.285	0.023
SMC3	structural maintenance of chromosomes 3	-0.284	0.026
BHLHE40	basic helix-loop-helix family member e40	-0.282	0.047
ITGB7	integrin subunit beta 7	-0.282	0.042
PRPF40A	pre-mRNA processing factor 40 homolog A	-0.282	0.015
SEPT7	septin 7	-0.282	0.025
G3BP1	G3BP stress granule assembly factor 1	-0.282	0.018
RB1	RB transcriptional corepressor 1	-0.281	0.021
XIAP	X-linked inhibitor of apoptosis	-0.281	0.019
ADD3	adducin 3	-0.281	0.015
CALR	calreticulin	-0.279	0.043
ATP2B1	ATPase plasma membrane Ca2+ transporting 1	-0.279	0.032
ZNF148	zinc finger protein 148	-0.279	0.023
RBBP7	RB binding protein 7, chromatin remodeling factor	-0.279	0.016
COPA	coatomer protein complex subunit alpha	-0.278	0.026
ACTR2	ARP2 actin related protein 2 homolog	-0.278	0.036
CLINT1	clathrin interactor 1	-0.277	0.010

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PJA2	praja ring finger ubiquitin ligase 2	-0.276	0.023
NBR1	NBR1, autophagy cargo receptor	-0.275	0.036
RAB8B	RAB8B, member RAS oncogene family	-0.275	0.043
PRKAA1	protein kinase AMP-activated catalytic subunit alpha 1	-0.274	0.017
UBA6	ubiquitin like modifier activating enzyme 6	-0.272	0.020
DDX3X	DEAD-box helicase 3, X-linked	-0.272	0.036
GLUD1	glutamate dehydrogenase 1	-0.272	0.023
PRRC1	proline rich coiled-coil 1	-0.271	0.027
VCP	valosin containing protein	-0.270	0.036
DEGS1	delta 4-desaturase, sphingolipid 1	-0.269	0.015
BCLAF1	BCL2 associated transcription factor 1	-0.268	0.025
RPN2	ribophorin II	-0.268	0.018
ZBTB33	zinc finger and BTB domain containing 33	-0.266	0.047
HSD17B4	hydroxysteroid 17-beta dehydrogenase 4	-0.265	0.015
IKZF3	IKAROS family zinc finger 3	-0.265	0.033
SMC1A	structural maintenance of chromosomes 1A	-0.265	0.026
ETS1	ETS proto-oncogene 1, transcription factor	-0.264	0.019
SSRP1	structure specific recognition protein 1	-0.264	0.043
ACTR3	ARP3 actin related protein 3 homolog	-0.264	0.026
DHX9	DEAH-box helicase 9	-0.263	0.014
SCAMP1	secretory carrier membrane protein 1	-0.262	0.025
DCTN1	dynactin subunit 1	-0.262	0.049
MTA2	metastasis associated 1 family member 2	-0.261	0.048
KIDINS220	kinase D-interacting substrate 220kDa	-0.260	0.024
TMBIM6	transmembrane BAX inhibitor motif containing 6	-0.260	0.039
MAP3K1	mitogen-activated protein kinase kinase kinase 1	-0.259	0.041
MCM3	minichromosome maintenance complex component 3	-0.259	0.044
ATRX	ATRX, chromatin remodeler	-0.258	0.032
CHD8	chromodomain helicase DNA binding protein 8	-0.258	0.021
UPF2	UPF2 regulator of nonsense transcripts homolog (yeast)	-0.257	0.026
STK38L	serine/threonine kinase 38 like	-0.257	0.042
GNAI3	G protein subunit alpha i3	-0.257	0.038
ACTB	actin beta	-0.257	0.046
ZEB2	zinc finger E-box binding homeobox 2	-0.257	0.034
G3BP2	G3BP stress granule assembly factor 2	-0.256	0.032
HNRNPU	heterogeneous nuclear ribonucleoprotein U	-0.256	0.011
STIP1	stress induced phosphoprotein 1	-0.255	0.032
IL2RG	interleukin 2 receptor subunit gamma	-0.255	0.023
UBE2J1	ubiquitin conjugating enzyme E2 J1	-0.255	0.043
GABPA	GA binding protein transcription factor alpha subunit	-0.255	0.036
YWHAG	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma	-0.255	0.021
DCAF7	DDB1 and CUL4 associated factor 7	-0.253	0.023
NARS	asparaginyl-tRNA synthetase	-0.252	0.042
TBL1XR1	transducin (beta)-like 1 X-linked receptor 1	-0.251	0.049
BAZ1B	bromodomain adjacent to zinc finger domain 1B	-0.251	0.021
USP9X	ubiquitin specific peptidase 9, X-linked	-0.250	0.045
VCPIP1	valosin containing protein interacting protein 1	-0.250	0.038
AASDHPPT	aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	-0.250	0.046

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