Genome-wide DNA methylation analysis in combat veterans reveals a novel locus for PTSD

Running Title - **Genome-wide DNA methylation in veterans with PTSD**

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Abstract

Objective: Epigenetic modifications such as DNA methylation may play a key role in the aetiology and serve as biomarkers for Posttraumatic stress disorder (PTSD). We performed a genome-wide analysis to identify genes whose DNA methylation levels is associated with PTSD.

Methods: A total of 211 individuals comprising Australian male Vietnam War veterans (n = 96) and males from a general population belonging to the Grady Trauma Project (n = 115) were included. Genome-wide DNA methylation was performed from peripheral blood using the Illumina arrays. Data analysis was performed using generalized linear regression models.

Results: Differential DNA methylation of 17 previously reported PTSD candidate genes was associated with PTSD symptom severity. Genome-wide analyses revealed CpG sites spanning *BRSK1, LCN8, NFG* and *DOCK2* genes were associated with PTSD symptom severity. We replicated the findings of *DOCK2* in an independent cohort. Pathway analysis revealed that among the associated genes, genes within actin cytoskeleton and focal adhesion molecular pathways were enriched.

Conclusions: These data highlight the role of DNA methylation as biomarkers of PTSD. The results support the role of previous candidates and uncover novel genes associated with PTSD, such as *DOCK2*. This study contributes to our understanding of the biological underpinnings of PTSD.

Significant Outcomes:

- These data highlight the role of DNA methylation as biomarkers of PTSD.
- We confirmed the role of previous PTSD candidates and identified novel loci associated with PTSD including *DOCK2*, a gene also implicated in Alzheimer's.
- These results add to the existing research and provide essential knowledge of the biological underpinnings of PTSD

Limitations:

- This is a cross sectional association study and provides a snapshot of the DNA methylation.
- The study may not have sufficient power to detect subtle DNA methylation changes.
- DNA methylation was assessed in blood hence causality cannot be inferred.

Introduction

Posttraumatic stress disorder (PTSD) is a debilitating disorder that occurs after exposure to a potentially life threatening traumatic event. About 70% of the population experience a traumatic event during their lifetime, however, only few individuals will go on to develop PTSD, suggesting there may be a genetic predisposition. Twin studies of Vietnam War veterans have confirmed that genetic factors contribute to risk of PTSD (1-3). Analyses of monozygotic and dizygotic twins showed that approximately 30% of the variance in liability for PTSD symptoms (re-experiencing, avoidance and hyper-arousal) is due to genetic factors (4-6). More recent twin studies suggest that heritable influences account for 46% of the variance in PTSD (7) but could be as high as 71% in females (8). Although both genetic and environmental risk factors for PTSD have been widely investigated, there are currently no biological factors that can be used to monitor at-risk individuals in real time (9, 10).

Genetic studies in PTSD have largely focused on candidate genes based on our current understanding of the neurobiology of PTSD (11). Many SNPs (single nucleotide polymorphisms) within candidate genes found to be associated with PTSD were within the promoter or non-coding regions and a complex interaction between genetic and environmental factors was suggested (9). In addition to candidate gene studies, recently a handful of exploratory genome-wide association studies in PTSD have also been reported. There have been several recommendations for PTSD GWAS studies (12-14) but to date only six PTSD GWAS studies have been performed in humans (15-20). The first GWAS study in PTSD detected association between the retinoid-related orphan receptor alpha (*RORA*) locus and PTSD in trauma exposed veterans (15). *RORA* has a role in protecting brain cells from injury, stress and disease (15) and the association with *RORA* has since been replicated in hurricane

exposed adults with traumatic stress symptoms (21). Subsequent GWAS studies identified the role of lincRNA *AC068718.1* (16) and *PRTFDC1* in PTSD (16, 22, 23).

While genetic associations in PTSD are of relevance, considering the complex interplay between genetic and environmental risk factors, focus has recently shifted to investigation of biological markers reflecting dynamic changes that are a hallmark of environmental modulation of the genome. Biological changes in response to stress occur rapidly and these changes and their sequelae likely depend on the developmental stage of the individual (24). Epigenetic modifications of DNA in response to changes in the environment induce changes in gene expression that are potentially reversible and that may be potential targets for novel therapies. One of the most common and widely studied epigenetic mechanisms is DNA methylation, which influences gene expression. DNA methylation is the addition of a methyl group at 5' position of the cytosine in the cytosine–phosphate-guanine dinucleotide (CpG) sites in the genome. DNA methylation levels have been associated with psychosocial stress, childhood trauma, and PTSD (25). We and others have identified DNA methylation differences in PTSD that are significantly associated with a history of childhood abuse (26, 27).

A few studies in humans have demonstrated the epigenetic contribution to PTSD. These have largely been focused epigenetic studies looking at methylation in targeted genes (28). An important study in humans found that early life trauma affects epigenetic regulation of hippocampal glucocorticoid receptor expression (29). A recent study investigated epigenetic regulation of the *BDNF* gene in PTSD development among veterans exposed to combat in the Vietnam War and observed higher DNA methylation of the *BDNF* promoter and PTSD diagnosis in combat-exposed individuals (30).

To our knowledge only three genome scans of methylation status in PTSD have been published (31-33). The first genome-wide methylation analysis reported an over-representation of immune system function genes that were hypomethylated in PTSD patients (33). A recent study

showed that DNA methylation of *HDAC4* (histone deacetylase 4), was associated with PTSD, fear-potentiated startle and functional connectivity in amygdala in women and this was driven by estrogen levels (32).

The aim of this study was to understand and quantify the contribution of DNA methylation to disease progression and severity of PTSD. Here, we investigated both candidate genes previously reported to be associated with PTSD and we performed a exploratory genome-wide scan to identify DNA methylation loci associated with PTSD vulnerability. Utilization of a homogenous cohort of age-matched male veterans all exposed to the Vietnam War is likely to yield new and consistent findings to uncover the biological underpinnings of PTSD.

Methods

Participant Recruitment

The samples comprise of a large cohort of veterans who have been or are currently being treated for PTSD at the Keith Payne Unit (KPU) within the Greenslopes Private Hospital (GPH). This population of older veterans is an ideal population for this study as they are accessible, motivated to participate and are of an age at which long lasting enduring epigenetic manifestations of trauma and PTSD are likely to be measurable. The Vietnam Veterans study at the Gallipoli Medical Research Foundation (GMRF), based at the GPH has systematically collected comprehensive clinical data, including psychological and physical health diagnoses and combat-trauma exposure, from a large representative cohort of Vietnam veterans (N = 299) (34, 35). Interview-based data are supplemented by pre-military and military data from Army records, including information about combat exposure. Half the veterans currently experience PTSD symptoms and 25% meet DSM-5 (The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition) diagnostic criteria for PTSD.

Genome-wide DNA methylation analysis was conducted in a total of 96 individuals selected from the Vietnam veteran participants. Cases were combat-exposed veterans with current PTSD (n = 48) and controls were veterans with no current PTSD and no previous PTSD diagnosis (n = 48). Care was taken to match the case-control groups for environmental factors (such as trauma history) and other demographics.

Each participant gave written informed consent before commencement of data collection. Ethics approval for the project was obtained from the Human Research Ethics Committee of the Queensland University of Technology and Greenslopes Private Hospital. This study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Clinical Assessments

All participants received clinical assessments by an experienced psychiatrist at the KPU at GPH based on DSM-5 criteria. Severity of PTSD symptoms was assessed by clinical psychologists using the Clinician-Administered PTSD Scale for DSM-5 (CAPS-5) (36) which is the "gold standard" for PTSD assessment. Higher scores reflect increased PTSD severity. For the current study we selected veterans with current PTSD diagnosis based on CAPS-5 (n = 48) and veterans with no current and no previous PTSD diagnosis (n =48) as cases and controls, respectively. Common comorbidities were assessed using the Mini International Neuropsychiatric Interview DSM IV (MINI), an instrument designed to assess major Axis 1 disorders with high validity and reliability (37, 38). The Depression Anxiety Stress Scale 21 (DASS-21) which is a self-report scale comprised of three subscales representing three different constructs: stress, depression and anxiety was also used (39). Higher scores for DASS-21 reflect increased symptoms of depression, anxiety and stress, respectively. The Cronbach's α for total DASS-21 was high with an $\alpha = 0.95$. The Connor-Davidson Resilience Scale (CD RISC) was used to measure resilience via a range of strategies that have been shown to be successful mediators in dealing with adversity (40). The scale has sound psychometric properties, (41). Higher scores indicate higher resilience and Cronbach's α was high: $\alpha = 0.92$. Structured clinical history included demographic information, smoking, diet and exercise, lifetime history of alcohol consumption, past and current illnesses, medications and family medical history. Information was also obtained related to military and combat such as term of service, number of times deployed, role and duration in the defence force.

Experimental Procedures

Blood samples were sent to the Australian Genome Research Facility (AGRF) for DNA extraction, DNA methylation and genotyping on microarrays. Upon arrival, samples were

stored at -20°C. Genomic DNA was extracted from a 2 ml blood sample, using MACHERY-NAGEL NucleoSpin L (MACHEREY-NAGEL GmbH & Co. KG, Dueren, NRW, Germany). Quality of the DNA was assessed through resolution on a 0.8% agarose gel at 130 V for 60 minutes. Samples were normalised to 200 ng of DNA in 4 µl.

For DNA methylation, samples were bisulphite converted with Zymo EZ DNA Methylation kit as previously described (27, 42). The Infinium platform assays more than 850,000 CpG sites, encompassing 99% of RefSeq genes. It covers 96% of CpG islands with multiple sites in the island, the shores (within 2 kb from CpG islands) and the shelves (> 2 kb from CpG islands). It also covers CpG sites outside of CpG islands and DNase hypersensitive sites as well as incorporating miRNA promoter regions.

Blood samples were sent to the Australian Genome Research Facility (AGRF) for RNA extraction and expression analysis. Upon arrival, samples were stored at -20°C. RNA was extracted from a 2.5 ml PaxGene (PreAnalytiX GmbH, Switzerland) blood sample, using MACHERY-NAGEL NucleoSpin RNA Blood (MACHEREY-NAGEL GmbH & Co. KG, Dueren, NRW, Germany). The quality and quantity of human total RNA was ascertained on the Agilent Bioanalyser 2100 using the NanoChip protocol. A total of 500ng of total RNA was then prepared for hybridisation to the Ilumina HumanHT 12 Expression Beadchip (array content HumanHT-12_V4_0_R2_15002873_B) as outlined in the Illumina manual and scanned in the Illumina iScan Reader.

Genotyping was performed using the Illumina PsychArray-24 BeadChip scanned with the Illumina iScan systems.

Statistical analysis

Raw beta values from EPIC Illumina arrays were exported into R (https://www.r-project.org/) for statistical analysis. For quality control (QC), pairs of technical and biological replicates

were included to test and confirm the robustness of the microarray procedures and all replicate correlations were high ($r > 0.97$), indicating high reproducibility. Samples with probe detection call rates < 95% as well as those with an average intensity value of either <50% of the experiment-wide sample mean or < 2000 arbitrary units (AU) were excluded from further analysis. The level of methylation was determined by calculating a "β value" - the ratio of the fluorescent signals for the methylated versus unmethylated sites. Intensity read outs, normalization and methylation beta values calculations were performed using the minfi Bioconductor R package version 1.10.2. For methylation analysis, IDAT files were loaded into the R (2.15) environment. The arrays were then background and control normalized using the minfi package (43). Subset-quantile Within-Array Normalisation (SWAN) was used to remove technical differences between Infinium I and Infinium II probes available in the minfi package (44, 45). The methylation status for each probe was recorded as a β-value that ranged between 0 and 1, where values close to 1 represent high levels of methylation and where values close to 0 represent low levels of methylation. A detection *P*-value was calculated for all probes on all arrays. A *P*-value>0.05 indicates that the data point is not significantly different from background measurements. Probes with >50% of the samples with a detection *P*-value>0.05, probes located on either the X or Y chromosomes and, probes with single-nucleotide polymorphisms present within 10–50 bp from query site, and within <10 bp from query site were removed. These resulted in a total of n = 866802 CpG probes. Finally, using recent annotations from Pidsley et al, 2016 (44) we removed probes whose signal could be affected by cross-hybridization or underlying genetic variation (n = 142370). These resulted in a total of n = 724432 CpG probes that were used for all subsequent analyses. Data were analysed using an established analysis pipeline comprising of custom statistical programs and scripts (27, 46, 47) written in R and Linux. Surrogate variable analyses (SVA) revealed 17 significant SVA vectors that were used as covariates in the model to correct for technical artifacts and

hidden confounds (48). Generalized regression models were performed to identify genomic sites significantly associated with PTSD, after correcting for the SVAs. For the genome-wide analysis, we employed the commonly used 10% false discovery rate (FDR) to account for multiple testing. For other candidate analysis, we used a gene-wise Bonferroni correction to account for the number of CpGs tested across each gene.

Gene expression data were transformed and normalized using the variance stabilizing normalization (49). Probes passing the filter criteria of Illumina probe detection p-value of < 0.05 in at least 5% of the samples were used for subsequent analysis. For this study, we extracted the DOCK2 gene probe for the post-hoc analysis and excluded one individual that failed quality checks hence we analyzed the DOCK2 gene expression for 95 veterans. We used SVA method to account for known and unknown factors such as batch effects by including them as covariates in the model, controlling for potential bias (48); SVA of gene expression revealed 13 SVA's and these were used as covariates in the regressions. Data were analysed using an established analysis pipeline (27, 46, 47).

Genotypes were obtained from the Illumina Psych array for all the 96 veterans with DNA methylation data. Initial quality control was performed in PLINK 1.9 (50, 51) - SNPs with >1% missing values, a Hardy-Weinberg equilibrium *P*-Value < 0.0001 and a minor allele frequency of < 5% were excluded, resulting in 302,607 genotyped SNPs. The genotypes were recoded as vcf files before uploading to the Michigan Imputation Server (https://imputationserver.sph.umich.edu/start.html#!pages/home) using SHAPEIT to phase haplotypes, and Minimac with the most recent 1000 Genomes reference panel (phase 3, version 5). Imputed genotypes were then filtered and recoded in PLINK removing samples with >5% missing values, and multi-allelic and monomorphic SNPs, SNPs >1% missing values, a Hardy-Weinberg equilibrium P < 0.0001, a minor allele frequency of < 5%. This resulted in 2,934,941 variants (2.9 million) in the imputed set of genotypes. For cis mQTL analysis, we identified

the cis interval as 1 Mb and tested SNPs 1Mb upstream or downstream of the gene. We performed association analysis using the methylation levels as a trait and the additive genotype for the SNP in PLINK. Functional annotation of genes was performed using the KEGG database via the Webgestalt interface (52) to identify enriched pathways with at least five genes within the pathway using a hypergeometric test for enrichment evaluation analysis and Bonferroni-adjusted *P*-value of 0.01.

Replication cohort and methods

Replication was performed using the Grady Trauma Project (GTP), details of the cohort are described in Gillespie et al (53). Briefly, the GTP is a large study conducted in Atlanta, Georgia, USA, that investigates the role of genetic and environmental factors in shaping responses to stressful life events. The GTP includes more than 7,000 participants from a predominantly African-American, urban population of low socioeconomic status (53). This population is characterized by high prevalence and severity of trauma over the lifetime and is thus particularly relevant for examining the effects of stressors on epigenetic markers. From the large GTP samples, genome-wide DNA methylation data was available for a subset, as described previously (54), and from this for replication we used data from115 males that were matched for adult trauma levels (all had two or more types of adult trauma). Adult trauma was assessed via the Trauma Events Inventory (TEI) (55). The TEI was the primary measure of non-child abuse trauma in the GTP as described before (27, 56). For PTSD Symptom Severity, the PTSD Symptomatic Scale (PSS) was used, which was the primary measure of PTSD symptoms in the GTP. We have previously shown that the PSS in the Grady samples is significantly associated with CAPS (27, 46). Additionally, information on childhood abuse was available for this cohort as was used as a covariate in the regression model. The GTP samples

were run on the Illumina 450k arrays as detailed in Zannas et al. (54) and Maddox et al. (32). Methylation beta values after corrections for batch effects were used for the analysis.

Results

Demographics

The study sample comprised a total of 96 Australian male veterans from the Vietnam War. Of the veterans, 82.3% were enrolled in the Army, 14.6% in the Airforce and 3.1% in the Navy. The veterans were aged between 62 and 88 years, with a mean age of 68.67 [0.45] years. This sample as a whole had normal cognitive functioning, with a mean Montreal cognitive assessment score of 26.14 [0.27], indicating a normal range of cognitive functioning. Details of the demographics including current age, age at start of service, PTSD symptom severity, cognitive assessment, resilience, depressive, anxiety and stress scores are presented in Table 1a. Based on the Clinician Administered PTSD Scale (CAPS) diagnosis, a total of 48 individuals had a current diagnosis of PTSD and 48 individuals did not meet the criteria for PTSD diagnosis. No significant differences in demographics including age, BMI, smoking, substance and alcohol abuse, suicide ideation, marital status, current employment status and medication status were observed across the two groups (Table 1b). As expected, the veterans with PTSD had significantly higher PTSD symptom severity scores and depression, anxiety and stress scores as per the DASS-21 scales.

DNA methylation of PTSD candidate genes

We systematically tested candidate genes that were reported to be associated with PTSD (11), to assess whether DNA methylation of the genes was associated with PTSD symptoms. To increase the power to detect biological differences across these groups, we focused our analysis on the quantitative PTSD symptom severity scores based on the CAPS-5. Details of the number of genes tested, the number of CpG sites within the gene on the Human EPIC array and the DNA methylation results are given in Table 2 and results are shown in Supplementary Table 1. Of the 35 genes, 30 genes had at least one CpG tagging the gene that was present on the

EPIC array. A total of 24 of 30 genes tested (81%) had at least one CpG site that was significantly associated with PTSD symptom severity (*P*-value < 0.05). Of these, 17 genes (57% of those tested) remained significant even after applying multiple testing correction.

Genome-wide DNA methylation in PTSD among combat veterans

We performed a genome-wide methylation analysis to identify CpG sites whose DNA methylation was significantly associated with PTSD symptom severity. We regressed PTSD symptom severity score against the DNA methylation levels, correcting for the SVAs. A total of five CpGs remained significant, after applying multiple testing correction (Table 3, Figure 1 and Supplementary Figure 1). These CpGs spanned the *BRSK1, NGF, LCN8* and *DOCK2* genes, one CpG site was intergenic (Figure 2).

Using genetic data available from the same individuals, we tested if the observed DNA methylation differences were genetically driven. We performed methylation quantitative trait analysis (mQTLs) by regressing the DNA methylation levels against the genotypes, adjusting for PCs. Specifically, for the CpGs spanning the *BRSK1, NGF, LCN8* and *DOCK2* genes, we checked for cis SNPs, i.e. SNPs within +/- 1Mb from the gene. For *BRSK1, NGF* and *LCN8* no cis-mQTLs were significant after multiple testing corrections. For *DOCK2*, we tested 2314 SNPs that were +/- 1Mb from the *DOCK2* gene and identified 5 SNPs that were significantly associated with DNA methylation levels (*P* < 2.16e-5). Four of these SNPs were in high linkage disequilibrium, details of these mSNPs are provided in Supplementary Table 2 and Supplementary Figure 2a. *DOCK2* mSNPs within both linkage disequilibrium blocks were also significantly associated with PTSD symptom severity (*P* –value = 0.01). Next, we categorized the DOCK2 DNA methylation results by the DOCK2 genotype for the 5 mSNPs. We identified that while the mSNPs significantly influenced the levels of DNA methylation of the DOCK2,

there were no differences in PTSD symptom severity within the genotype strata ($p > 0.05$, Supplementary Figure 2b).

Replication of the DOCK2 DNA methylation in the Grady Trauma Project

We sought to replicate the DNA methylation results in an independent cohort of primarily African-Americans from a general population of suburban Atlanta, Georgia, USA, belonging to the Grady Trauma Project (GTP) (53). Using 115 males from the GTP cohort, we assessed whether DNA methylation was significantly associated with PTSD symptom severity using the same approach as in the discovery study above. Of five genome-wide significant CpGs, data from three CpG sites were present in the GTP cohort that was run on the 450k Illumina methylation array. Of these, the DOCK2 CpG (cg16277944) was also significantly associated with PTSD symptom severity also in the GTP ($P = 0.0028$). Moreover, this remained significant even after adjusting for childhood abuse levels assessed using the Childhood Trauma Questionnaire (CTQ).

Post-hoc analysis of DOCK2 gene expression

Next, we performed post-hoc analysis of DOCK2 gene expression in the discovery veteran dataset for the same individuals from whom we had DNA methylation. Analysis showed that DNA methylation for the significant DOCK2 CpG (cg16277944) was negatively correlated with DOCK2 gene expression extracted from the Illumina HumanHT12-v4.0 array ($r = -0.21$, p-value =0.023, Supplementary Figure 3a). Moreover, the DOCK2 gene expression significantly distinguished the PTSD group from the non-PTSD group (p-value= 0.012 Supplementary Figure 3b).

Pathways

To identify molecular underpinnings of PTSD, we sought to investigate which biological pathways were overrepresented among the genes that were significantly associated with PTSD symptom severity. For this purpose, we chose genes that had a *P*-value < 0.005 in the above analysis of genome-wide DNA methylation in PTSD and investigated these via the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway (n = 337 genes). The significantly enriched pathways included regulation of actin cytoskeleton pathway (10 genes, 7-fold enrichment, adjusted *P*-value = 0.0001) and focal adhesion pathway (9 genes, 6-fold enrichment, adjusted *P*-value = 0.0006).

Conclusions

Individuals differ in their susceptibility towards PTSD and in recent years several risk factors for PTSD have been identified. Both candidate gene studies and GWAS studies have pointed towards several genes involved in vulnerability towards PTSD yet the biology of PTSD remains largely unexplored. Epigenetic modifications such as DNA methylation are one likely biological mechanism that might underlie PTSD and there is growing literature pointing towards the role of DNA methylation in PTSD (25). DNA methylation mediates gene expression of endocrine system components, signalling molecules, and transcription factors (57-59) and alters the efficacy of the upstream signalling cascades on gene expression and behaviour (60, 61). Since DNA methylation changes accumulate over time, the molecular signature of trauma exposure may be evident substantially before symptoms of a disorder develop. However, the timeline and permanence of trauma-induced epigenetic changes are unclear. Understanding how the epigenome responds to trauma may allow for early detection and effective intervention.

A genome-wide DNA methylation analysis in a cohort of veterans was performed to explore the extent to which DNA methylation contributes to the aetiology of PTSD. Compared to previous studies, this was a relatively homogenous cohort comprising age-matched male veterans who had been subjected to traumatic experiences of combat exposure during the Vietnam War. We demonstrated that differential DNA methylation of reported PTSD candidate genes were associated with PTSD symptom severity in this cohort of veterans, implying a role of epigenetics in PTSD susceptibility. This included genes such as the *RORA* gene that was identified via a genome-wide association study in PTSD (15), hence our study provides the first evidence that DNA methylation changes within *RORA* are significantly associated with PTSD symptoms. Furthermore, we have previously shown that a variant of the *NOS1AP* gene is associated with PTSD (35) and here we provide further evidence of the role of the *NOS1AP*

gene in PTSD at the DNA methylation level. We were unable to find any evidence for some genes including *ADRB2, PRTFDC1* and *NLGN1*, however these results must be interpreted with caution as some genes were tagged by few CpG sites on the array, we are limited to one tissue and it is likely that our sample is underpowered and/or mechanisms other than DNA methylation might play in role in PTSD susceptibility for these genes. For instance, DNA methylation is a complex, flexible and dynamic epigenetic mechanism (62, 63) and is often coupled with equally important active DNA demethylation processes (64). DNA methylation of *FKBP5* decreases *FKBP5* gene and protein expression that occurs following glucocorticoid binding to GR, and demethylation can be induced by prolonged glucocorticoid exposure (26, 65). Therefore, demethylation at some of the reported genes post-trauma may prime transcriptional responses to subsequent stimuli and future studies are needed to further examine the methylation/demethyation dynamics in PTSD. Additional studies exploring the relationship between DNA methylation and other epigenetic processes such as histone modifications will also help to decipher mechanisms driving gene expression in PTSD.

At the genome-wide level, we identified 5 CpG sites spanning 4 genes (*NGF, DOCK2, BRSK1* and *LCN8*) the DNA methylation levels of which were significantly associated with PTSD symptom severity after corrections for multiple testing. Within these genes, we also observed other CpG sites that were significant, albeit at the nominal level. For the CpG site within the *DOCK2*, we were also able to replicate this finding in an independent cohort from the general population, highlighting the robustness of these findings. Querying a public dataset (66) revealed that DNA methylation of the DOCK2 CpG site was highly correlated between blood and brain regions (prefrontal cortex: r = 0.44, P-value = 7.8e-5, entorhinal cortex: r = 0.28, P-value = 0.019 and superior temporal gyrus: r = 0.45, P-value = 5.7e-5, Supplementary Figure 4). Post-hoc analysis of gene expression in veterans revealed a significant negative correlation between the gene expression and DNA methylation levels of *DOCK2*, which is expected given

that the *DOCK2* CpG lies in the 5'UTR region of the gene close to the promoter region. Moreover, *DOCK2* gene expression was significantly different between the PTSD and non-PTSD groups, providing functional evidence of *DOCK2* DNA methylation changes. The DOCK family plays an important role in development and functioning of neurons, linked to neuropsychiatric and neurodegenerative disorders. The *DOCK2* (dedicator of cytokinesis 2) gene is highly expressed in the immune system (67) and the brain, and has been implicated in neuroinflammation of the Alzheimer's disease pathology (68). The DOCK2 is a key molecule contributing to innate immune activation and amyloid beta plaque burden in Alzheimer's (69). Interestingly, PTSD resulting from military service has been associated with a doubled risk of cognitive decline and dementia resulting from Alzheimer's disease (70, 71), however the pathomechanisms for this are unknown (72). Pathway analyses revealed that genes associated with PTSD symptom severity were significantly enriched within the "regulation of actin cytoskeleton" and "focal adhesion" pathways. Both actin cytoskeleton and focal adhesion processes play a crucial role in synaptic plasticity and neurodegeneration, thereby again highlighting a likely link between neurodegeneration and PTSD. To the best of our knowledge, this is the first reported association of *DOCK2* gene with PTSD, and replication in larger independent studies is warranted. Moreover, the role of genetic regulation of this locus warrants further study.

Since epigenetic processes such as DNA methylation are dynamic in nature and highly sensitive to environmental triggers, there is great promise that appropriate environmental, psychological and pharmacological interventions could help counteract or reverse the negative effects of trauma, building resilience through changes in gene activity. Future directions include large prospective studies where DNA methylation and gene expression is measured at different time points across the lifespan of an individual, to uncover DNA methylation trajectories of health and disease. One common problem associated with PTSD is the

persistence of DNA methylation marks following traumatic events. Recent studies have explored the relationship between DNA methylation, histone modification and long-term trajectories in gene regulation, including transgenerational inheritance of DNA methylation marks (73). Moreover, studies that are designed to differentiate between DNA methylation marks before PTSD exposure, rather than those which are a consequence of PTSD will allow cause from effect to be distinguished. Early predictive biomarkers identified in high-risk individuals are particularly interesting, given their potential clinical utility.

This study has several limitations. We have performed a cross sectional association study and it would be interesting to have a more informative longitudinal study, possible pre and post-combat. Also, this is a small study hence we acknowledge that we may not have sufficient power to detect subtle DNA methylation changes, nevertheless, the homogeneity of the cohort with regards to age and trauma-matched male veterans is a major strength of this study. Moreover, we were able to replicate the results in an independent general population of different ethnicity, pointing towards the generalizability and reproducibility of these findings. Nevertheless, replication and validation of these findings in larger and diverse samples is warranted. Finally, we have assessed DNA methylation in blood and given the tissue specificity it is difficult to infer causality from these results. Nevertheless, for the DOCK2 CpG site, using a public dataset, we observed a high concordance in DNA methylation from blood and brain regions (66).

It is of utmost importance to validate genes associated with PTSD using functional tests as demonstrated by several studies that have tested the association of PTSD-related genetic variants with brain structure and function. For instance, Almli et al identified a genome-wide SNP that was associated with PTSD and was also found to be a methylation quantitative trait locus. This risk variant was also associated with decreased medial and dorsolateral cortical activation to fearful faces (22). In another study, a SNP (rs2267735) in ADCYAP1R1, the gene

encoding PACAP receptor1 (PACR1), was associated with PTSD in females (74). The PTSD risk allele at this SNP was found to be associated with expression of PACR1 in human brain, increased amygdala reactivity to threat stimuli, reduced amygdala/hippocampal connectivity, and decreased hippocampal activation during contextual fear conditioning by fMRI (74-76). Future studies are required to reveal the biological relevance of peripheral DNA methylation differences observed in PTSD by linking these with functional data such as neuroimaging and neurophysiological measures. Dissecting how peripheral epigenetic marks correlate with methylation and gene expression in brain tissue will allow a deeper understanding of the involvement of the genes in PTSD etiology (77).

Additionally, studies aimed at understanding neural circuits will also provide valuable insights into the neural mechanisms underlying PTSD. Characteristic changes in neural circuits including alterations in the hippocampus, amygdala and cortical regions such as the anterior cingulate, insula and orbitofrontal regions play a direct role in PTSD development (78, 79). The three major neural circuits implicated in PTSD include the fear learning circuitry, context processing circuitry and emotion regulation circuitry (80). Awareness of neural circuits in PTSD is vital given that there are targeting treatments aimed at specific dysregulated neural circuits e.g. pharmacological intervention using d-cycloserine, a partial NMDA agonist, combined with exposure therapy facilitates fear extinction in phobic patients (81). Integrating information about the neural circuits together with other comprehensive knowledge such as genetic, neuroendocrine and environmental information holds potential for improved, personalized and integrated pharmacological and psychological treatment in PTSD (82).

In summary, this study has provided evidence of the role of DNA methylation in the molecular aetiology of PTSD. Our genome-wide analyses have implicated several novel loci, including *DOCK2* gene that provides an indication of common susceptibility genes involved in both

PTSD and neurodegenerative disorders and might reveal likely therapeutic targets for PTSD. Advances in PTSD research will be facilitated by large international collaborative efforts such as the establishment of the recent Psychiatric Genomics Consortium Posttraumatic Stress Disorder Workgroup (83). These consortia will allow researchers to harness data from larger, in-depth characterized and diverse samples, allowing for identification of robust biomarkers for PTSD. Better understanding of the biological underpinnings of combat and other stress associated disorders such as PTSD will pave the way for improved diagnosis, early intervention and treatment.

Acknowledgements

The authors thank the Gallipoli Medical Research Foundation for their support, especially Miriam Dwyer and Dr Sarah McLeay for their project management support. The authors would also like to acknowledge Dr Madeline Romaniuk for psychological expertise and Dr John Gibson and the team at the Keith Payne Unit, and the staff and investigators at Greenslopes Private Hospital for their valuable contribution to the study. All authors extend their gratitude to the study participants for their generous provision of data and time. The PTSD Initiative was funded by the Queensland Branch of the Returned & Services League of Australia (RSL QLD). The Gallipoli Medical Research Foundation wishes to thank the RSL QLD for their generous donation, and Sullivan Nicolaides Pathology and Queensland X-Ray for their in-kind support. The authors thank QUT, and IHBI for financial and administrative support. Divya Mehta was funded via the QUT Vice Chancellors Research Fellowship. Dagmar Bruenig was funded by a Gallipoli Medical Research Foundation PhD Scholarship.

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Tables

Figures Legends

Figure 1: DNA methylation in PTSD - Manhattan plot depicting CpG sites across the chromosomes whose DNA methylation was significantly associated with PTSD Symptom severity. The 5 CpGs above the blue line remained significant at 10% fdr.

Figure 2: Boxplots of DNA methylation changes in PTSD - The veterans are grouped into PTSD and non-PTSD to highlight the group differences. For CpGs spanning BRSK1, NGF, LCN8 and DOCK2 genes, decreased DNA methylation (hypomethylation) was associated with increased PTSD symptom severity while for the intergenic CpG hypermethylation was associated with increased PTSD symptom severity.

Supplementary Information

Supplementary Table 1 – DNA methylation in previously reported PTSD candidate genes

Supplementary Table 2 – List of the 5 cis-SNPs associated with *DOCK2* cg16277944 DNA methylation levels (cis-mQTLs)

Supplementary Figure 1 – Quantile-quantile (QQ) plots of the genome-wide analysis of DNA methylation with PTSD symptom severity reveals no inflation and a lambda of 1.03

Supplementary Figure 2a – Linkage disequilibrium plot of the DOCK2 mSNPs using the Broad Institute SNAP website (http://archive.broadinstitute.org/mpg/snap/index.php) using the 1000 genomes CEU data. SNP rs169082 is in high linkage disequilibrium (R2>0.80) with rs10036335, rs192358 and rs264877 as indicated by the dotted line.

Supplementary Figure 2b – Boxplots of DOCK2 mQTLs, stratified by genotypes indicated that there were no significant genotype x PTSD symptom severity interactions (p>0.05) for all the 5 mSNPs.

Supplementary Figure 3a – Scatterplot of DOCK2 gene expression and DNA methylation in the discovery cohort of veterans (r = -0.21, p-value = 0.023) shows a negative correlation.

Supplementary Figure 3b – Boxplot of DOCK2 gene expression is significantly different in the PTSD and non-PTSD groups in the discovery cohort of veterans (p-value = 0.012).

Supplementary Figure 4 – comparison of DNA methylation levels of DOCK2 CpG site in blood and brain using a public data resource (http://epigenetics.iop.kcl.ac.uk/bloodbrain/). This online tool allows to correlate DNA methylation in blood with four brain regions (prefrontal cortex, entorhinal cortex, superior temporal gyrus and cerebellum) from 71-75 matched samples for all probes present on the Illumina 450K Beadchip array.